DISSECTING THE GENETIC BASIS OF VARIOUS ADAPTATION TRAITS IN ALFALFA USING QTL MAPPING

by

LAXMAN ADHIKARI

(Under the Direction of Ali Mekki Missaoui)

ABSTRACT

Fall dormancy (FD) and winter hardiness (WH) influence seasonal yield, stand persistence, and latitudinal adaptation of alfalfa (*Medicago sativa* L.). Selection of dormant alfalfa genotypes with higher WH has been a common practice. This research was carried out to dissect the genetic basis of FD and WH through quantitative trait loci (QTL) mapping and explore the potential of incorporating WH in non-dormant alfalfa. Other traits, including time of flowering (TOF), spring yield (SY), cumulative summer biomass (CSB), and leaf rust resistance were also evaluated. An F1 population was derived for linkage analysis and QTL mapping by crossing a dormant winter-hardy cultivar (3010, ♀) with a non-dormant cold-sensitive cultivar (CW 1010, ♂). Genotyping-by-sequencing was used for single nucleotide polymorphism (SNP) marker discovery. Dormancy and WH were evaluated according to NAAIC protocols. We mapped 45 FD and 35 WH QTLs on the genetic linkage maps of both parents. More than 70% of the FD QTLs did not share genomic locations with WH QTLs, suggesting that the two traits are inherited separately. This study also showed that using late autumn to early winter regrowth height is more reliable than early autumn in estimating alfalfa dormancy in southern environments with mild-winters. The QTL markers with higher phenotypic effects (R^2) can be

used in marker-assisted selection (MAS) of non-dormant alfalfa with improved WH. Incorporating WH in non-dormant alfalfa can ensure forage production in late autumn and early winter to minimize the forage gaps. In this research, we mapped a total of 25 QTLs for TOF, 17 QTLs for SY, six QTLs for CSB, and eight QTLs for leaf rust resistance in the same alfalfa population. Four TOF QTLs were detected in corresponding genomic positions of flowering QTLs of *M. truncatula* reported previously. The multiple QTLs detected for leaf rust resistance suggests that alfalfa resistance to the rust pathogen is polygenic. The QTL markers identified in this study constitute an important addition to alfalfa genomic resources and can be validated in populations with diverse genetic backgrounds and in multiple environments for potential use in MAS.

INDEX WORDS: *Medicago*, alfalfa, adaptation traits, fall dormancy, winter hardiness, cold tolerance, genotyping-by-sequencing (GBS), SNPs, genetic map, pseudo-testcross, quantitative trait loci (QTL), flowering time, spring yield, leaf rust, UNEAK, LOD,

DISSECTING THE GENETIC BASIS OF VARIOUS ADAPTATION TRAITS IN ALFALFA USING QTL MAPPING

by

LAXMAN ADHIKARI

B.Sc.Ag., Tribhuvan University, Nepal, 2009

M.S., Oklahoma State University, 2014

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

DISSECTING THE GENETIC BASIS OF VARIOUS ADAPTATION TRAITS IN ALFALFA USING QTL MAPPING

by

LAXMAN ADHIKARI

Major Professor:	Ali Mekki Missaoui
Committee:	Chung-Jui Tsai
	Dayton Wilde
	Orville M. Lindstrom
	Paul Schliekelman

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2018

DEDICATION

For my late father Prem Prasad Adhikari who passed away when I was in my PhD first year. My academic journey started in a semi-urban village in Nepal and I am here today just because of him. And for my mom, beloved wife, lovely daughter, brothers and sisters for love, support and encouragement.

ACKNOWLEDGEMENTS

I can't thank enough the persons who directly and indirectly assisted me in the completion of this project. I would like to express my deepest gratitude and thanks to my advisor Dr. Ali M. Missaoui, for mentoring me for the past four and half years. He is the friendliest, helpful and encouraging person I ever found in my educational journey. He has given me the freedom to pursue different projects and assisted me until they come to an end point. I am truly thankful to my committee members, Prof. Dr. Chung-Jui Tsai, Prof. Dr. Orville M. Lindstrom, Prof. Dr. Dayton Wilde, Dr. Paul Schliekelman for their guidance along this journey. This project would not have completed without the regular support and effort of Jonathan Markham, who had supervised especially the field and the greenhouse works. I wish to express my sincere thanks to Jonathan. I am grateful to my coolest friends Dr. Dev Paudel, Dr. Shiva Makaju and Dr. Sameer khanal for their assistance in data analysis. I would especially like to thank my master adviser Prof. Dr. Yaqi Wu from Oklahoma State University and my previous supervisor Prof. Dr. Thomas Juenger from The University of Texas at Austin, who showed me a path that has a single end point named professional scientist. I can't express in words how much I am thankful to Er. Yek R. Adhikari, my brother, for believing and supporting me since my childhood to today in my every step.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS V

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

Alfalfa fall dormancy (FD) 3

Winter hardiness (WH) 4

Relationship between FD and WH 5

Indoor screening for cold tolerance and WH 7

Winter-hardy alfalfa in Georgia and the southeast 7

Alfalfa timing of flowering (TOF) and spring yield (SY) 8

Alfalfa leaf rust 10

GBS, linkage and QTL mapping in alfalfa 10

References 14

2 DISSECTING KEY ADAPTATION TRAITS IN THE POLYPLOID PERENNIAL *MEDICAGO SATIVA* USING GBS-SNP MAPPING 20

Abstract 21

Introduction 22

Materials and Methods 26

Results 33

Discussion 43

Tables and Figures 50
References 68
Appendix: Supplemental Figures 74
3 DISSECTING GENOTYPIC AND PHENOTYPIC VARIATIONS IN SPRING FLOWERING TIME AND BIOMASS YIELD IN ALFALFA 90
Abstract 91
Abbreviations 92
Introduction 92
Materials and Methods 96
Results 98
Discussion 104
Conclusion 109
Tables and Figures 110
References 122
4 QUANTITATIVE TRAIT LOCI MAPPING OF LEAF RUST RESISTANCE IN TETRAPLOID ALFALFA 126
Highlights 127
Abstract 127
Abbreviations 129
Introduction 129
Materials and Methods 131
Results 134
Discussion 138

Conclusion 141

Tables and Figures 143

References 152

5 RELEVANCE OF INDOOR FREEZING TOLERANCE TEST TO PREDICT ALFALFA WINTER HARDINESS 156

Introduction 156

Materials and Methods 158

Results 160

Discussion 162

Conclusion 164

Tables and Figures 165

References 167

6 SUMMARY 169

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Alfalfa (*Medicago sativa* L.), the queen of forages, is a perennial herbaceous legume widely grown for livestock feed. It is a cool season, C3, and a highly heterozygous autotetraploid (2n=4x=32) with genome size of one Gb and tetrasomic inheritance [1,2]. It is a versatile crop primarily used for hay, pasture, silage, green-chop, and pellets [3]. Alfalfa and its mixtures constitute the largest source of hay in the United States and they have been planted in over seven million hectares in 2017 [4]. Argentina, Canada, Italy, Russia and China are the other major alfalfa producers [5]. When grown with grasses such as bermudagrass, orchardgrass or timothy, alfalfa provides quality pasture for grazing animals [6]. Alfalfa is a highly nutritious forage which is primarily rich in protein and total digestible nutrients (TDN) [7]. It also fixes atmospheric nitrogen at the rate of 130-220 lbs per acre per year [8], which eventually gets incorporated into the plant protein [7]. This minimizes the requirement of nitrogen application from other sources. Alfalfa possesses a deep rooting system which enhances soil structure, prevents soil erosion and serves as an important component of sustainable cropping systems when grown as a cover crop [1]. Alfalfa is known for heavy phosphorus uptake, which is essential in arid and semi-arid regions [9]. The number of alfalfa cuts per year depends mostly on the climate of the region. In the southeast USA where the winters are often mild, alfalfa can be harvested in spring, summer and fall seasons. Stem cutting is a common vegetative propagation method in alfalfa, where the cuttings start quick rooting if the donor is young and vigorous. Alfalfa is a self-incompatible species pollinated naturally by insects such as honey bee, bumble

bee, leaf-cutter bee, and alkali bee that can trip the flower for pollination [6]. Mass selection and recurrent selection are the most common breeding approaches used for alfalfa improvement [10]. Available alfalfa cultivars are mostly synthetics [6].

The presence of abundant wild type alfalfa in southeast Asia, near the southern Caucasus Mountains, possibly indicates the region as the center of origin of alfalfa [6,1]. As an exotic species, alfalfa was introduced into the Americas sometime in the 16th Century [1]. Hybridization between two alfalfa sub-species *M. sativa spp. sativa* and *M. sativa spp. falcata* takes place serenely, and has the same karyotype [6]. Alfalfa related *Medicago* species forming the same gene pool, *M. glomerata* and *M. prostrata*, have yellow flowers and coiled pods and exist in the form of diploid species [11]. However, *M. prostrata* also exists at the tetraploid level [11]. Alfalfa is closely related to the model legume *M. truncatula*, and they possess structurally and functionally similar genomic regions [12]. As a model species, the *M. truncatula* has a reference genome and has been utilized in alfalfa genomic studies. For example, when constructing genetic linkage maps, alfalfa chromosomes are assigned based on the marker positions on the *M. truncatula* reference genome [13].

Alfalfa is often cultivated in rotation with cereals such as wheat and oat [6]. However, its growth can be impacted by allelopathic effects of the previously grown crops (e.g. cereal rye), if it is planted immediately after the allelopathic crop [4]. Several biotic factors such as diseases and insects are major challenges to alfalfa production. Over 100 fungal species have been identified that are pathogenic diseases in alfalfa, such as leaf rust, anthracnose, downy mildew, stem rot, leaf spot, and root rot [6]. Bacterial wilt and alfalfa mosaic virus are other alfalfa disease factors other than fungi. Root knot galls caused by various *Meloidogyne* nematode spp. destroy alfalfa swards [14]. Major insect-pests reported on alfalfa are alfalfa weevil, alfalfa

aphid, pea aphid and potato leafhopper [6]. Salinity, drought, freezing, low pH, and excessive aluminum are common abiotic stresses in alfalfa production [15]. Alfalfa adaptation traits such as winter hardiness and alfalfa fall dormancy have been considered crucial for its latitudinal distribution and can be enhanced via genetic and genomic approaches. Winter-hardy non-dormant alfalfa can fill up the seasonal forage gap via extending the growing season [16].

Alfalfa fall dormancy (FD)

Fall dormancy (FD) is an important adaptation trait in alfalfa that influences the latitudinal distribution of the species [16]. With decreasing temperature and photoperiod in autumn, some alfalfa accessions suspend their growth, and become dormant [5]. Nevertheless, morphological differences in alfalfa with various dormancy levels could be realized at different time points in different latitudes. For instance, in Georgia and neighboring states with often mild winters, alfalfa dormancy is more prominent in late fall and winter [17]. Since regrowth height is the key indicator for FD level in alfalfa [18,19], FD is assessed using a regression equation derived from the relationship of FD levels of the standard checks and plant regrowth height after autumn clipping. At the field scale, proper FD assignment requires data from at least two seasons and multi-location trials [19].

Investigating alfalfa FD started in 1940's when scientists evaluated alfalfa strains for recovery patterns after the first and second autumn clipping [20]. They found a constant FD dormancy expression in alfalfa. Effect of FD on average annual yield was estimated for six alfalfa varieties with dormancy levels one to six, which showed a notable difference between the yield of dormant and non-dormant for the first and second cuttings in central Oregon environments [21]. However, they did not observe significant differences in mean yield recorded for five years under three-cut regime. Wang et al. (2009), also did not find significant

differences in average annual dry matter yield and dormancy levels in a temperate environment, and hence suggested not to choose FD levels as the main criteria for alfalfa cultivar improvement [22]. Nevertheless, they reported earlier shoot re-growth of non-dormant than dormant alfalfa cultivars after harvesting. Alfalfa genotype interactions with the environment were observed for 19 different cultivars with various FD classes when tested in multi-location trials [23]. Recently, FD associated microRNAs (miRNA) were identified using high-throughput small RNA (sRNA) sequencing in the dormancy check cultivars Maverick and CUF 101 [24]. Transcriptome profiling using RNA-seq analysis enabled the detection of some putative alfalfa FD genes with differential expression [25,5].

Winter hardiness (WH)

Winter hardiness (WH) in alfalfa refers to its ability to withstand harsh winter factors such as freezing temperature, frost-heaving, diseases, moisture level, low light intensity, and snow [26,27]. It is a quantitative trait, and the winter-hardy alfalfa undergoes dormant without getting damaged and have higher freezing tolerance [28]. Winter-hardy germplasm is important for cultivation in northern climates because of higher stand persistence, yield and quality [28]. WH is the consequence of various physiological, molecular, and cellular processes. For instance, carbon, nitrogen and lipid metabolism alters cell's ability to tolerate the harsh winter environment, especially freezing temperature [28]. The other factors that affect WH include cold acclimation potential, plant vigor, disease resistance ability, root and crown structure, and FD level [28].

Investigation of alfalfa WH started in the earlier 20th century. In 1929, J. L. Weimer, a USDA scientist, investigated the factors associated with alfalfa winter killing and found that factors such as soil moisture and the amount of insulation the soil environment provided to the

roots affects alfalfa winter hardiness. Later research on alfalfa WH has primarily focused on molecular, cellular and physiological approaches. Mohapatra et al. (1988) reported the role of ABA induced genes associated with cold acclimation in alfalfa [29]. The cold acclimation-specific gene (cas18) was identified in a diploid alfalfa species (*M. falcate* cv Anik) [30]. The cas18 is a hydrophilic protein with glycine and threonine as major amino acids of the polypeptide chain. Cold-acclimated cells had about 30-fold higher expression of cas18 gene than the non-acclimated cells [30]. Past studies also reported induction of cold acclimation-specific gene cas15 in alfalfa after cold treatment. Moreover, accumulation of different levels of carbohydrates in the roots and crowns was observed to be one of the determining factors of cold tolerance in alfalfa [31,32]. Recent study reported how the cold acclimation of freezing tolerant and freezing sensitive alfalfa differ at the proteomic level [33]. They observed several changes in proteins related to other physiological processes such as photosynthesis and metabolism to generate cold acclimation in freezing tolerant genotype.

Relationship between FD and WH

The most investigated adaptation traits in alfalfa are FD and WH. The relationship between alfalfa FD and WH has been discussed in several reports. In 1960, Dale Smith observed more winter injury in the alfalfa with greater fall height, and also found decreased seed yield in dormant lines [34]. Similarly, a positive phenotypic correlation between WH, fall growth, and rapid recovery after clipping were discussed in [35]. Perry and McIntosh (1986) found phenotypic correlation and genetic linkage between FD and cold hardiness in alfalfa, and such genetic linkage hindered selecting tall fall regrowth with cold hardiness [18]. Negative relation between alfalfa fall yield and winter survival impeded simultaneous selection of alfalfa for higher fall yield and better winter survival [36]. Selecting alfalfa with low fall regrowth for

higher winter survival also resulted in low spring-summer yield. Cunningham et al. (1998) investigated the physiological and biochemical basis of FD related WH in alfalfa, and found that alfalfa with contrasting FD had different levels of winter survival because of the acclimation difference in buds and roots [37].

However, Brummer et al. (2000) found an opposite relationship than previously described, between winter injury and autumn growth in alfalfa, where they found a weak negative phenotypic as well as genotypic correlations between autumn height and winter injury in a population derived from ABI408 (*M. sativa*, clone) x WISFAL-6 (*M. falcata*, clone) [38]. They also suggested that concurrent improvement in both fall growth and WH in alfalfa is possible, and the relationship between these traits in alfalfa is more dependent on the type of germplasm evaluated. Similarly, Haagenson et al. (2003) documented that a semi-dormant germplasm '98-132' acquired similar winter injury as the dormant cultivar 'Vernal' and the '98-132' also had the equivalent amount of root sugar concentrations as other dormant cultivars [39]. The report also indicated that generating less fall dormant and winter-hardy alfalfa could be possible by better understanding of physiological and molecular mechanisms controlling these two traits. The idea of concurrently improving fall regrowth (especially fall forage yield) and WH in alfalfa has been further endorsed with Weishaar et al. (2005) experiment, where they showed the potential of improving WH in non-dormant alfalfa using recurrent selection based entirely on winter injury [40]. Recent reports based on genetic analysis also evidenced no or poor genetic linkages between FD and WH in alfalfa. Li et al. (2015) reported separate QTLs for FD and winter injury and argued for independent manipulation of the two traits [41]. We also reported QTLs for WH and FD, mostly in separate genomic regions, with the potential of simultaneous improvement of alfalfa two adaptation traits [17].

Indoor screening for cold tolerance and WH

Freezing tolerance is an important factor in determining alfalfa WH, which is very important for alfalfa cultivation in cold temperate climates [28]. The freezing stress tolerant plants often have higher adaptation and persistence in the field [10]. Thus, selection for freezing tolerance could be a selection for WH in alfalfa. Selecting the freezing tolerant genotypes in a controlled environment for eventual WH improvement is also common in other grasses, such as in perennial ryegrass (*Lolium perenne* L.) [42]. Alfalfa selected indoor using a walk-in freezer for enhanced freezing tolerance exhibited a better winter survival in the field, and thus selected plants could be used as parents for WH improvement breeding program [10]. In a past study, alfalfa population with higher freezing tolerance were achieved after recurrent selection in indoor environments [43]. Further, the molecular changes that were related to alfalfa cold acclimation were observed when alfalfa was recurrently selected for freezing tolerance in an artificial environment. For instance, the alfalfa cold related genes, such as cold-acclimation specific gene 15 (*cas15*) and galactinol synthase (GaS), were induced when the breeding germplasms were kept in unheated greenhouse [44]. Therefore, indoor selection method can be used as a reliable phenotyping method to improve alfalfa winter hardiness because occurrences of winters to test plants in the field is uncertain, as winter is mostly unpredictable [10].

Winter-hardy alfalfa in Georgia and the southeast

In winter, temperature in the southeastern varied from severe to mild depending on the latitude and elevation. For example, when Appalachian Mountains got sub-freezing temperatures with snow blindness, Florida (FL) would have > 25 °C and sunny weather [45]. Therefore, regionally tested cold hardy alfalfa are important for southeastern region, except FL. The colder temperature and snow are often observed in winters of the northern states of the southeast USA.

In Georgia, the alfalfa genotypes that can tolerate occasional freezing and have regrowth potential in mild winter are preferred because winter in the northeastern, mountain part of Georgia such as Blairsville, exhibit an average high temperature of around 10 ºC and an average minimum temperature of around -8 ºC in winter months [46]. In this region, the below freezing temperature persists for several days in the winter. Because of the cold winter condition in the southeast, developing winter-hardy alfalfa genotypes that can tolerate freezing winter is important for enhancing forage biomass yield. Further, identifying molecular markers and QTLs related to alfalfa WH can be useful for marker-assisted selection (MAS), because the field selection is tedious due to unpredictable weather and requires data for multi-years and multi-locations. The MAS can be utilized for developing winter-hardy alfalfa genotypes suitable for the region. Also, the WH trait can be incorporated into desirable germplasms, such as non-dormant to extend the growing season.

Alfalfa timing of flowering (TOF) and spring yield (SY)

TOF is an important index for harvesting, as farmers often harvest alfalfa at first bloom stage [6]. Like other perennials, total growing degree days (GDD) requirement for alfalfa to reach the flowering stage differs between growing seasons as well as latitudes of the growing regions [47]. Harvest time can be managed by manipulating flowering traits in alfalfa. For instance, if harvesting fresh forage after dormant season (earlier in the spring) is the goal, then, early flowering alfalfa accessions must be selected for breeding. Similarly, if early to mid-spring frost stress are problematic for the region, then selecting late flowering alfalfa accessions could be a choice to delay the harvesting. Delayed flowering could also be associated with higher biomass, but the quality of forage has to be considered before making a decision. There is limited information regarding the genetic and genomic basis of alfalfa TOF. Some alfalfa flowering

related genes were investigated using transformation approach. Yuehui et al. (2013) isolated alfalfa FRIGIDA-Like gene, *MsFRI-L*, and performed RT-PCR to observe the expression pattern of the gene in alfalfa organs [48]. The transgenics Arabidopsis with *MsFRI-L* expression had delayed flowering under long day conditions. Expression of alfalfa CCCH-type zinc finger protein gene *MsZFN* in Arabidopsis also resulted in delayed flowering transgenics [49]. Transcripts levels of different flowering genes such as FT, FLC, SOC1 and GI were changed in the transgenics. The QTLs for flowering time in *M. truncatula* were mapped previously, which can be used for comparative analysis of TOF in alfalfa [50].

Spring is one of the favorable seasons for alfalfa growth as the optimum temperature for its growth is 15-25 °C which is characteristic of most of the temperate regions [51]. Typically, the newer seedlings require higher temperature for establishment than a fully established alfalfa plant. Spring yield (SY) is an important source of fresh forage because most of the perennial grasses undergo dormant in winter, and growing winter annuals are difficult because of frequent snow and frost damage, especially in the northern latitudes. Alfalfa SY was positively correlated with summer and fall yield in a previous study [36]. Finding the genetic basis and genomic regions controlling SY in alfalfa is valuable to enhance the biomass of locally adapted cultivars. So far, limited information is available for QTL mapping of SY in alfalfa. However, a few reports dissecting genomic positions controlling alfalfa biomass are available. Alfalfa biomass related marker loci were previously reported using RFLP and SSR markers [52]. Li et al. (2011), identified at least 15 SSR markers strongly associated ($p < 0.005$) with alfalfa yield [53]. In a recent study, biomass related consensus genomic regions were identified in multiple *Medicago* population [54].

Alfalfa leaf rust

Alfalfa leaf rust caused by *Uromyces striatus* has been reported in Georgia nearly six decades ago [55]. The disease is common in late summer and fall. Rust damages foliage part of alfalfa and becomes more damaging upon delayed harvesting. Alfalfa spores overwinter in the southern states and re-infect plants once the favorable environment comes [56]. Warm, hot and damp weather is favorable conditions for alfalfa rust pathogen spread. Rust infected hay may also cause allergic reactions in livestock and the hay has a low digestibility [57]. To the best of our knowledge, there are no reports on genetic and genomic analysis of alfalfa leaf rust using QTL mapping.

GBS, linkage and QTL mapping in alfalfa

Genotyping-by-sequencing (GBS), an approach to prepare library for sequencing for SNP discovery and genotyping, is based on genome digestion with methylation sensitive restriction enzymes for reducing complexity [58]. The GBS procedure is cost effective, robust and straightforward, and has also become popular for species without reference genomes and polyploids with complex genome such as alfalfa [13]. Even other autopolyploid, e.g. potato [59], and allopolyploid, e.g. switchgrass [60] have been tested successfully for the GBS approach. The GBS discovered SNPs have enabled the genomic studies, such as genome-wide association (GWAS), genomic selection (GS) and genetic linkage mapping on several crops. In tetraploid alfalfa, Li et al. (2014) successfully used the GBS method and discovered 3591 SNP markers for genetic linkage mapping [13]. Recent genomic studies on alfalfa also used GBS successfully [61,62]. Rocher et al. (2015) indicated that GBS is one of the appropriate approaches for discovering SNPs in polyploid species like alfalfa [61].

Genetic linkage mapping in alfalfa has previously been conducted with traditional markers such as AFLP, RFLP and SSR [1], and the maps were mostly unsaturated and were not detailed at the level of sub-genomes. Brouwer and Osborn (1999) constructed linkage map on tetraploid alfalfa with single-dose restriction fragments (SDRFs) markers identified using backcross populations, where they had seven linkage groups (LG) with 88 loci [63]. The AFLP and SSR based genetic linkage maps of tetraploid alfalfa had a low marker density (a marker per 7-9 cM) [64]. Alfalfa genetic maps constructed using SSR markers obtained from expressed sequence tags (ESTs) and bacterial artificial chromosome (BAC) inserts was relatively denser but unsaturated [65]. Nonetheless, recently Li et al. (2014) constructed linkage maps of tetraploid alfalfa with GBS SNPs, where each parental maps had a higher marker density (1-1.5 cM/marker) and fully saturated [13]. The LGs identified in that study were mostly syntenous with *M. truncatula* genome [13].

Tetraploid alfalfa has tetrasomic inheritance and the loci segregated with complex pattern in subsequent generations. Therefore, only certain biallelic markers that are segregated to specific patterns are usable for constructing genetic linkage maps of tetraploid alfalfa [13]. Some recent programs especially designed for autotetraploid e.g. TetraploidSNPMap [66] can handle biallelic markers segregating in simplex (abbb x bbbb), duplex (aabb x bbb) and double-simplex (abbb x abbb) patterns [13]. However, the program is not adjustable to work with thousands of SNPs. Linkage mapping in autopolyploids can also be performed with an F1 population derived from two heterozygous parents using a method which uses the single dose allele (SDA) markers unique to each parent that are segregated in 1:1 (ab x aa) [13]. The markers that are segregating as in the testcross (1:1), i.e. segregating for only one parent, are known as testcross markers and the strategy that used these markers for linkage mapping is known as pseudo-testcross strategy

[67]. Using this strategy linkage maps can be constructed with the software designed for diploids such as JoinMap [68]. Nevertheless, the pseudo-testcross strategy uses only a portion of markers from the genome, but still useful for species with complex genomes and marker segregation ratios, and the method has been successfully used in various species [67]. Recently, Paudel et al. (2018) used this strategy successfully to construct first highly-saturated genetic linkage map of allopolyploid napiergrass [69].

Alfalfa valuable traits such as biomass yield, drought, FD and winter injury/survival were primarily the focus of previous QTL studies. Brouwer et al. (2000) mapped QTLs for fall growth, freezing injury and winter hardiness, and concluded that using predictor trait for WH could be efficient in the absence of WH data [70]. They also suggested that genetic factors controlling FD and WH can be manipulated separately. Robins et al. (2005) mapped QTLs for alfalfa biomass in a full sib, F1 population, using single marker analysis [52]. They detected 41 associated marker alleles for alfalfa biomass production. Recently, Ray et al. (2015) identified a total 25 QTLs for alfalfa biomass yield under drought stress using backcross (BC1) mapping populations [71]. Also, an association mapping identified significantly associated ($P < 0.005$) alfalfa loci for crude protein and minerals traits [72]. A genome-wide association study conducted with GBS SNPs identified 36 loci associated with alfalfa salt tolerance during germination [73]. Alfalfa fall regrowth height and winter injury were mapped using F1 population using RFLP, SSR and SNP markers led to the identification of multiple QTLs for the fall regrowth height and winter injury [41]. However, most of the detected QTLs spanned ~ 10 cM on the genome, indicating the necessity of further studies to narrow down the QTL region. Moreover, the linkage maps used for QTL mapping in alfalfa were primarily based on traditional markers and not at sub-genome level.

To our knowledge, the alfalfa adaptation and agronomic traits we mapped in this study were poorly studied or mapped on non-saturated genetic linkage maps. Consequently, mapping those traits on saturated alfalfa genetic maps will unravel the genomic positions of associated loci and opens the door for functional analyses of those genomic regions. Therefore, the objectives of this research were; i) to construct alfalfa high density genetic linkage maps using GBS SNPs ii) to map agronomically important alfalfa traits such as fall dormancy, winter hardiness, timing of flowering, spring yield, cumulative summer biomass, and leaf rust resistance iii) to screen alfalfa for cold temperature tolerance in an indoor freezer and find the relationship between indoor testing and field winter hardiness.

References

1. **Li X, Brummer EC** (2012) Applied genetics and genomics in alfalfa breeding. Agronomy 2 (1):40-61

2. **Adhikari L, Missaoui AM** (2017) Nodulation Response to Molybdenum Supplementation in Alfalfa and it's Correlation with Root and Shoot Growh in Low Ph Soil. Journal of Plant Nutrition 40:2290-2302. doi:10.1080/01904167.2016.1264601

3. **Smith S, Bouton J, Hoveland C** (1989) Alfalfa persistence and regrowth potential under continuous grazing. Agronomy Journal 81 (6):960-965

4. **Adhikari L, Mohseni-Moghadam M, Missaoui A** (2018) Allelopathic Effects of Cereal Rye on Weed Suppression and Forage Yield in Alfalfa. American Journal of Plant Sciences Vol.09No.04:685-700. doi:10.4236/ajps.2018.94054

5. **Zhang S, Shi Y, Cheng N, Du H, Fan W, Wang C** (2015) De Novo Characterization of Fall Dormant and Nondormant Alfalfa (Medicago sativa L.) Leaf Transcriptome and Identification of Candidate Genes Related to Fall Dormancy. PloS one 10 (3)

6. **Duke J (1981)** Handbook of legumes of world economic importance. New York , NY:Plenum Press

7. https://www.unce.unr.edu/publications/files/ag/other/fs9323.pdf

8. http://nmsp.cals.cornell.edu/publications/factsheets/factsheet39.pdf Cornell University. 2008

9. https://alfalfa.ucdavis.edu/+symposium/2010/files/talks/CAS20_Ottman AlfalfaFertilizer.pdf

10. **Castonguay Y, Michaud R, Nadeau P, Bertrand A** (2009) An indoor screening method for improvement of freezing tolerance in alfalfa. Crop science 49 (3):809-818

11. **Quiros CF, Bauchan G** (1988) The genus Medicago and the origin of the Medicago sativa complex. Alfalfa and alfalfa improvement 29

12. **Yang S, Gao M, Xu C, Gao J, Deshpande S, Lin S, Roe BA, Zhu H** (2008) Alfalfa benefits from Medicago truncatula: the RCT1 gene from M. truncatula confers broad-spectrum resistance to anthracnose in alfalfa. Proceedings of the National Academy of Sciences 105 (34):12164-12169

13. **Li X, Wei Y, Acharya A, Jiang Q, Kang J, Brummer EC** (2014b) A Saturated Genetic Linkage Map of Autotetraploid Alfalfa (Medicago sativa L.) Developed Using Genotyping-by-Sequencing Is Highly Syntenous with the Medicago truncatula Genome. G3 (Bethesda) 4 (10):1971-1979. doi:10.1534/g3.114.012245

14. **Reynolds HW, Carter WW, O'Bannon JH** (1970) Symptomless Resistance of Alfalfa to Meloidogyne incognita acrita. Journal of nematology 2 (2):131-134

15. https://openprairie.sdstate.edu/etd/1757/

16. **Adhikari L, Razar RM, Paudel D, Ding R, Missaoui AM** (2017) Insights into Seasonal Dormancy of Perennial Herbaceous Forages. American Journal of Plant Sciences Vol.08No.11:31. doi:10.4236/ajps.2017.811179

17. **Adhikari L, Lindstrom OM, Markham J, Missaoui AM** (2018) Dissecting Key Adaptation Traits in the Polyploid Perennial Medicago sativa Using GBS-SNP Mapping. Frontiers in Plant Science 9 (934). doi:10.3389/fpls.2018.00934

18. **Perry MC, McIntosh M, Wiebold W, Welterlen M** (1987) Genetic analysis of cold hardiness and dormancy in alfalfa. Genome 29 (1):144-149

19. **Teuber L, Taggard K, Gibbs L, McCaslin M, Peterson M, Barnes D** (1998) Fall dormancy. Standard tests to characterize alfalfa cultivars. North American Alfalfa Improve. Conf., 36th, Bozeman, MT, ate 1998. pp 2-6

20. **Sprague M, Fuelleman R** (1941) Measurements of recovery after cutting and fall dormancy of varieties and strains of alfalfa, Medicago sativa. Journal of the American Society of Agronomy

21. **Heichel G, Henjum K** (1990) Fall dormancy response of alfalfa investigated with reciprocal cleft grafts. Crop science 30 (5):1123-1127

22. **Wang C, Ma B, Yan X, Han J, Guo Y, Wang Y, Li P** (2009) Yields of alfalfa varieties with different fall-dormancy levels in a temperate environment. Agronomy journal 101 (5):1146-1152

23. **Xu G-m, Yan X-b, Wang C-z, Wang Y-h, Fan W-n** (2012) Performance of alfalfa varieties with different fall dormancy across different production areas of Henan Province of China. African Journal of Agricultural Research 7 (46):6197-6203

24. **Fan W, Zhang S, Du H, Sun X, Shi Y, Wang C** (2014) Genome-Wide Identification of Different Dormant Medicago sativa L. MicroRNAs in Response to Fall Dormancy. PLoS ONE 9 (12):e114612. doi:10.1371/journal.pone.0114612

25. **Zhang S, Wang C** (2014) Transcriptome profiling of gene expression in fall dormant and nondormant alfalfa. Genomics Data 2:282-284

26. **McKenzie JS, Paquin R, Duke SH** (1988) Cold and Heat Tolerance. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and Alfalfa Improvement. Agronomy Monograph, vol

29. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp 259-302. doi:10.2134/agronmonogr29.c8

27. http://www.canr.msu.edu/uploads/resources/pdfs/e2310.pdf

28. **Brouwer DJ, Duke SH, Osborn TC** (2000) Mapping Genetic Factors Associated with Winter Hardiness, Fall Growth, and Freezing Injury in Autotetraploid Alfalfa Research support provided by a Hatch grant from the College of Ag. and Life Sci., Univ. of Wisconsin-Madison. Crop Science 40 (5):1387-1396. doi:10.2135/cropsci2000.4051387x

29. **Mohapatra SS, Poole RJ, Dhindsa RS** (1988) Abscisic Acid-Regulated Gene Expression in Relation to Freezing Tolerance in Alfalfa. Plant Physiology 87 (2):468-473

30. **Wolfraim LA, Langis R, Tyson H, Dhindsa RS** (1993) cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, cas18, of alfalfa (Medicago falcata) cells. Plant Physiology 101 (4):1275-1282

31. **Castonguay Y, Nadeau P, Lechasseur P, Chouinard L** (1995) Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winterhardiness. Crop Science 35 (2):509-516

32. **Cunningham S, Nadeau P, Castonguay Y, Laberge S, Volenec J** (2003) Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting alfalfa germplasms. Crop Science 43 (2):562-570

33. **Chen J, Han G, Shang C, Li J, Zhang H, Liu F, Wang J, Liu H, Zhang Y** (2015) Proteomic analyses reveal differences in cold acclimation mechanisms in freezing-tolerant and freezing-sensitive cultivars of alfalfa. Frontiers in Plant Science 6:105. doi:10.3389/fpls.2015.00105

34. **Smith D** (1961) Association of fall growth habit and winter survival in alfalfa. Canadian Journal of Plant Science 41 (2):244-251

35. **Busbice T, Wilsie C** (1965) Fall Growth, Winterhardiness, Recovery after Cutting and Wilt Resistance in F2 Progenies of Vernal× DuPuits Alfalfa Crosses. Crop Science 5 (5):429-432

36. **Stout DG, Hall JW** (1989) Fall growth and winter survival of alfalfa in interior british columbia. Canadian Journal of Plant Science 69 (2):491-499. doi:10.4141/cjps89-060

37. **Cunningham S, Volenec J, Teuber L** (1998) Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. Crop Science 38 (4):962-969

38. **Brummer EC, Shah MM, Luth D** (2000) Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. Crop Science 40 (4):971-977

39. **Haagenson D, Cunningham S, Volenec J** (2003) Root physiology of less fall dormant, winter hardy alfalfa selections. Crop science 43 (4):1441-1447

40. **Weishaar MA, Brummer EC, Volenec JJ, Moore KJ, Cunningham S** (2005) Improving Winter Hardiness in Nondormant Alfalfa Germplasm. Crop Science 45 (1):60-65. doi:10.2135/cropsci2005.0060

41. **Li X, Alarcón-Zúñiga B, Kang J, Tahir N, Hammad M, Jiang Q, Wei Y, Reyno R, Robins JG, Brummer EC** (2015) Mapping fall dormancy and winter injury in tetraploid alfalfa. Crop Science 55 (5):1995-2011

42. **Waldron B, Ehlke N, Vellekson D, White D** (1998) Controlled freezing as a indirect selection method for field winterhardiness in turf-type perennial ryegrass. Crop science 38 (3):811-816

43. **Castonguay Y, Dubé MP, Cloutier J, Bertrand A, Michaud R, Laberge S** (2013) Molecular physiology and breeding at the crossroads of cold hardiness improvement. Physiologia plantarum 147 (1):64-74

44. **Castonguay Y, Bertrand A, Michaud R, Laberge S** (2011) Cold-induced biochemical and molecular changes in alfalfa populations selectively improved for freezing tolerance. Crop Science 51 (5):2132-2144

45. https://climate.ncsu.edu/edu/SETemp

46. http://weather.uga.edu/index.php?content=calculator&variable=CC&site=WATUGA

47. **Sharratt BS, Sheaffer CC, Baker DG** (1989) Base temperature for the application of the growing-degree-day model to field-grown alfalfa. Field Crops Research 21 (2):95-102. doi.org/10.1016/0378-4290(89)90045-2

48. **Chao Y, Yang Q, Kang J, Zhang T, Sun Y** (2013) Expression of the alfalfa FRIGIDA-Like Gene, MsFRI-L delays flowering time in transgenic Arabidopsis thaliana. Molecular Biology Reports 40 (3):2083-2090. doi:10.1007/s11033-012-2266-8

49. **Chao Y, Zhang T, Yang Q, Kang J, Sun Y, Gruber MY, Qin Z** (2014) Expression of the alfalfa CCCH-type zinc finger protein gene MsZFN delays flowering time in transgenic Arabidopsis thaliana. Plant Science 215-216:92-99.doi.org/10.1016/j.plantsci.2013.10.012

50. **Pierre J-B, Huguet T, Barre P, Huyghe C, Julier B** (2008) Detection of QTLs for flowering date in three mapping populations of the model legume species Medicago truncatula. Theoretical and Applied Genetics 117 (4):609-620. doi:10.1007/s00122-008-0805-4

51. http://learningstore.uwex.edu/assets/pdfs/A3681.PDF

52. **Robins JG, Luth D, Campbell TA, Bauchan GR, He C, Viands DR, Hansen JL, Brummer EC** (2007) Genetic mapping of biomass production in tetraploid alfalfa. Crop science 47 (1):1-10

53. **Li X, Wei Y, Moore KJ, Michaud R, Viands DR, Hansen JL, Acharya A, Brummer EC** (2011) Association Mapping of Biomass Yield and Stem Composition in a Tetraploid Alfalfa Breeding Population. The Plant Genome 4 (1):24-35. doi:10.3835/plantgenome2010.09.0022

54. **Ray IM, Monteros MJ, Julier B, Sledge MK, Brummer EC** (2018) Identification of Consensus Regions Associated with Shoot Biomass Production in the Medicago Genome. Crop Science 58 (3):1037-1060. doi:10.2135/cropsci2017.11.0657

55. Service E-UAr (1960) Index of plant diseases in the United States. US Department of Agriculture

56. https://ipm.illinois.edu/diseases/rpds/301.pdf

57. https://cropwatch.unl.edu/2018/rust-alfalfa

58. **Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE** (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS one 6 (5):e19379

59. **Bastien M, Boudhrioua C, Fortin G, Belzile F** (2018) Exploring the potential and limitations of genotyping-by-sequencing for SNP discovery and genotyping in tetraploid potato. Genome 61 (6):449-456. doi:10.1139/gen-2017-0236

60. **Lu F, Lipka AE, Glaubitz J, Elshire R, Cherney JH, Casler MD, Buckler ES, Costich DE** (2013) Switchgrass Genomic Diversity, Ploidy, and Evolution: Novel Insights from a Network-Based SNP Discovery Protocol. PLOS Genetics 9 (1):e1003215. doi:10.1371/journal.pgen.1003215

61. **Rocher S, Jean M, Castonguay Y, Belzile F** (2015) Validation of Genotyping-By-Sequencing Analysis in Populations of Tetraploid Alfalfa by 454 Sequencing. PLoS ONE 10 (6):e0131918. doi:10.1371/journal.pone.0131918

62. **Biazzi E, Nazzicari N, Pecetti L, Brummer EC, Palmonari A, Tava A, Annicchiarico P** (2017) Genome-wide association mapping and genomic selection for alfalfa (Medicago sativa) forage quality traits. PloS one 12 (1):e0169234

63. **Brouwer D, Osborn T** (1999) A molecular marker linkage map of tetraploid alfalfa (Medicago sativa L.). Theoretical and Applied Genetics 99 (7-8):1194-1200

64. **Julier B, Flajoulot S, Barre P, Cardinet G, Santoni S, Huguet T, Huyghe C** (2003) Construction of two genetic linkage maps in cultivated tetraploid alfalfa (Medicago sativa) using microsatellite and AFLP markers. BMC Plant Biology 3:9-9. doi:10.1186/1471-2229-3-9

65. **Sledge MK, Ray IM, Jiang G** (2005) An expressed sequence tag SSR map of tetraploid alfalfa (Medicago sativa L.). Theoretical and Applied Genetics 111 (5):980-992. doi:10.1007/s00122-005-0038-8

66. http://www.bioss.ac.uk/knowledge/tetraploidmap/

67. **Wu S, Yang J, Huang Y, Li Y, Yin T, Wullschleger SD, Tuskan GA, Wu R** (2010) An improved approach for mapping quantitative trait Loci in a pseudo-testcross: revisiting a poplar mapping study. Bioinformatics and biology insights 4:1-8

68. https://www.kyazma.nl/index.php/JoinMap/Evaluate/

69. **Paudel D, Kannan B, Yang X, Harris-Shultz K, Thudi M, Varshney RK, Altpeter F, Wang J** (2018) Surveying the genome and constructing a high-density genetic map of napiergrass (Cenchrus purpureus Schumach). Scientific reports 8 (1):14419

70. **Brouwer DJ, Duke SH, Osborn TC** (2000) Mapping genetic factors associated with winter hardiness, fall growth, and freezing injury in autotetraploid alfalfa. Crop Science 40 (5):1387-1396

71. **Ray IM, Han Y, E L, Meenach CD, Santantonio N, Sledge MK, Pierce CA, Sterling TM, Kersey RK, Bhandari HS, Monteros MJ** (2015) Identification of Quantitative Trait Loci for Alfalfa Forage Biomass Productivity during Drought Stress. Crop Science 55 (5):2012-2033. doi:10.2135/cropsci2014.12.0840

72. **Jia C, Wu X, Chen M, Wang Y, Liu X, Gong P, Xu Q, Wang X, Gao H, Wang Z** (2017) Identification of genetic loci associated with crude protein and mineral concentrations in alfalfa (Medicago sativa) using association mapping. BMC plant biology 17 (1):97-97. doi:10.1186/s12870-017-1047-x

73. **Yu L-X, Liu X, Boge W, Liu X-P** (2016) Genome-Wide Association Study Identifies Loci for Salt Tolerance during Germination in Autotetraploid Alfalfa (Medicago sativa L.) Using Genotyping-by-Sequencing. Frontiers in Plant Science 7 (956). doi:10.3389/fpls.2016.00956

CHAPTER 2

DISSECTING KEY ADAPTATION TRAITS IN THE POLYPLOID PERENNIAL *MEDICAGO SATIVA* USING GBS-SNP MAPPING

Adhikari L, Lindstrom OM, Markham J, Missaoui AM (2018). Frontiers in Plant Science 9 (934). doi:10.3389/fpls.2018.00934. Reprinted here with permission of the publisher.

Abstract

Understanding key adaptation traits is crucial to developing new cultivars with broad adaptations. The main objective of this research is to understand the genetic basis of winter hardiness (WH) and fall dormancy (FD) in alfalfa and the association between the two traits. QTL analysis was conducted in a pseudo-testcross F1 population developed from two cultivars contrasting in FD (3010 with FD = 2 and CW 1010 with FD = 10). The mapping population was evaluated in three replications at two locations (Watkinsville and Blairsville, GA). FD levels showed low to moderate correlations with WH (0.22-0.57). Assessing dormancy in winter is more reliable than in the fall in southern regions with warm winters. The mapping population was genotyped using Genotyping-by-sequencing (GBS). Single dose allele SNPs (SDA) were used for constructing linkage maps. The parental map (CW 1010) consisted of 32 linkage groups spanning 2127.5 cM with 1377 markers and an average marker density of 1.5 cM/SNP. The maternal map (3010) had 32 linkage groups spanning 2788.4 cM with 1837 SDA SNPs with an average marker density of 1.5 cM/SNP. Forty-five (45) significant ($P < 0.05$) QTLs for FD and 35 QTLs for WH were detected on both male and female linkage maps. More than 75 % (22/28) of the dormancy QTL detected from the 3010 parent did not share genomic regions with WH QTLs and more than 70% (12/17) dormancy QTLs detected from CW 1010 parent were localized in different genomic regions than WH QTLs. These results suggest that the two traits have independent inheritance and therefore can be improved separately in breeding programs.
Keywords**:** alfalfa, genetic map, QTL, genotype x environment interaction, fall dormancy, winter hardiness

Introduction

Alfalfa (*Medicago sativa* L.) is a perennial cool-season forage legume grown worldwide for hay, pasture and silage [1,2]. It is native to the southwestern and central Asia, near southern Caucasus Mountains [2,3]. Alfalfa is well-regarded for providing high-quality forage with high protein content and nutritive value [2]. Like other legumes, alfalfa fixes atmospheric nitrogen (N), up to 130-220 lbs per acre per year, thereby supplying N to itself and succeeding crops in rotation [4]. In the US., alfalfa and its mixtures contribute a major part of haylage production, where the productivity varies from 1.1 ton/acre (Rhode Island) to 7 ton/acre (California) with an average national productivity of 3.45 ton/acre in 2016 [5]. Alfalfa is cross-pollinated and highly heterozygous. It is a polyploid (2n = 4x = 32) with tetrasomic inheritance and a genome size near 1 Gb [3]. Alfalfa grows best in cool sub-tropical and warm temperate environments [2]. Growth and yield are remarkably affected by seasonal dormancy and low temperature stress in winter [6]. Alfalfa evolved FD as an important adaptation strategy to survive in latitudes with harsh winter conditions. The short growth cycle of fall-dormant alfalfa varieties limits not only the amounts of biomass accumulated but also the seasonal distribution, which is reduced to a few harvests per year in summer. FD rating (FDR) of alfalfa cultivars is assigned based on fall regrowth height, after clipping, to 11 groups ranging from FD 1 to FD 11 with lower numbers indicating more dormant [7]. These groups are very dormant, (FD 1, 2); dormant (FD 3, 4), moderately dormant (FD 5), semi-dormant (FD 6, 7), non-dormant (FD 8, 9), and very non-dormant (FD 10, 11) [8]. Dormancy classes are assigned based on standard check cultivars. Diminishing day length and temperature in the fall season are the two major environmental factors triggering physiological dormancy in alfalfa [9,10]. FD is a strongly expressed trait where certain genotypes exhibit slow growth leading to a short stature and decumbent plant architecture

after autumn clipping [6,7]. In order to assign FD accurately in the field, it is suggested to collect information from multiple locations for least for 2 years [7].

The genetic control of FD in alfalfa is not known and the investigation into the endogenous factors influencing FD will be valuable for developing cultivars with no- or short-fall dormancy. The molecular basis of dormancy has been studied mostly in woody species adapted to temperate environments. There are few reports on QTLs associated with the dormancy trait in herbaceous forage species. Some QTLs associated with fall growth and WH were mapped using an interspecific hybrid population developed by crossing annual x perennial ryegrass [11]. Day length and temperature are most likely the two major environmental cues that plants use to sense the environmental changes [12,13]. Genomic studies have identified a number of genes involved in the control of dormancy induction and growth cessation, including circadian clock regulators [14]. However, Mckenzie et al. (1988) argued that alfalfa FD is not physiologically similar to that of higher trees since the plant exhibits dormancy due to decreasing day length and temperature, but it is reversible when alfalfa is switched to an environment with warmer temperature and longer photoperiod. The research investigating the genetic and physiological basis of FD in alfalfa, in the context of genes, quantitative trait loci (QTL) and hormones regulating the process of alfalfa FD [15,3] suggested that FD in alfalfa is correlated with winter survival and very often, fall dormant alfalfa is considered more winter-hardy [16,17]. In northern latitudes, mostly dormant germplasm is grown because they have better chances of completing the development cycle and go dormant before the onset of freezing temperatures in early fall. There is a lack of consensus regarding the relationship between fall regrowth and WH even though alfalfa breeders have been routinely using FD as a surrogate to select for cold tolerance in northern latitudes. A strong phenotypic as well as the genetic correlation between

dormancy and WH was observed in alfalfa breeding populations developed from wide dormancy crosses involving parents with contrasting dormancy ratings [18]. Cunningham et al. (1998) examined the impact of selection for differences in FD on carbohydrate and protein accumulation in roots and crown buds as well as its effect on winter survival and bud development in four alfalfa parents and their progeny [19]. They concluded, after three cycles of selection, that imposing selection on FD will lead to improved cold acclimation and winter survival. Brummer et al. (2000) stressed the need for reexamining the relationship between FD and WH because contrary to the traditional concept, they found a weak association between the two traits [10]. Similarly, quantitative trait loci (QTLs) independently controlling autumn plant growth and winter survival were reported indicating the possibility of independent improvement of the two traits through marker-assisted selection (MAS) [18]. In a recent study, scientists have identified differentially expressed genes such as C-repeat binding factors (CBF) in response to freezing stress in alfalfa which may be induced regardless of the genotype dormancy [20]. Zhang et al. (2015) also observed several differentially expressed genes in fall dormant lines in leaf transcriptome analysis [21]. Similarly, alfalfa cold acclimation specific (*CAS*) genes such as *cas15* and other cold related genes are also potential genetic factors controlling WH without affecting dormancy [18,22]. There has been a limited progress in developing non-dormant alfalfa varieties with improved cold and freeze tolerance. Most of the studies have been conducted in Northern latitudes on dormant germplasm or in growth chambers rather than in the field under real winter conditions. Significant differences are known to exist between natural and artificial cold acclimation conditions and therefore plants that are cold acclimated in growth chambers may react differently compared to those acclimated naturally [23]. Field grown plants are often exposed to the varying light spectrum and intensities compared to the constant conditions in

growth chambers. Plants in the field are also frequently exposed to strong winds that influence gene expression and plant structure [24]. Dhanaraj et al. (2007) documented a large number of genes that were induced in a growth chamber, but not under field conditions [23]. An understanding of the interconnection between genetic factors and networks that control winter dormancy and WH will provide fundamental knowledge needed for the development of genomic resources that will enable selection of non-dormant alfalfa germplasm that persist well under occasional freezing temperatures. Therefore, dissecting the relationship between alfalfa FD and WH at the genomics level would be valuable to improving alfalfa.

Genetic analysis of FD and WH in alfalfa through QTL mapping requires adequate genome coverage with molecular markers. A large number of SNPs can be obtained cost effectively through next generation sequencing methods like genotyping-by-sequencing (GBS) even in species with no prior genome assemblies. The GBS method developed by Elshire et al. (2011) comprises selective fragmentation of DNA by specific enzymes, ligation of common and barcode adapters, PCR, clean up and sequencing [25]. The GBS method has been used successfully in discovering SNP markers in several diploids and autotetraploids crop species such as potato (*Solanum tuberosum* L.), rose (*Rosa hybrida*), and alfalfa [26-28]. However, in species with tetrasomic inheritance, only certain biallelic SNPs (simplex, duplex, double simplex) can be mapped. Since most of the mapping software are designed for diploid genomes, mapping autopolyploids is cumbersome. Some new software applications can handle this issue, but they still have limitations. TetraploidMap seems useful in adjusting markers segregating in various ratios (simplex, duplex, double simplex), but it can fit only about 800 markers and works better when each linkage group has less than 50 markers [29,28]. Similarly, TetraploidSNPMap can support a higher number of SNPs, but requires SNP dosage data from SNP array [30].

However, most of the autotetraploid QTL maps available so far such as potato [31] and alfalfa [18] maps were constructed using TetraploidMap or TetraploidSNPMap. Mapping autotetraploids with unique kinds of markers using software like JoinMap is also common. Often the pseudo-testcross simplex markers (AB x BB), i.e. markers heterozygous in one parent and not the other, are used to construct autotetraploid genetic maps in software like JoinMap [32]. The pseudo-testcross strategy allows the use of several thousand single dose SNPs and is considered a simple method of linkage mapping [28]. Identifying quantitative trait loci (QTLs) underlying FD and WH will enable understanding the genetic factors controlling these traits and helps in discovering markers associated with each trait. Manipulation of these alleles through MAS will enable the development of non-dormant alfalfa cultivars with improved WH. The objective of this study was to understand the genetic basis of alfalfa FD and WH via genetic linkage analysis and QTL mapping.

Materials and Methods

Mapping population

An F1 population was developed by crossing a tetraploid dormant (FD = 2) winter-hardy alfalfa cultivar (3010, ♀) with a tetraploid non-dormant (FD = 10) winter susceptible cultivar (CW 1010, ♂). The cross was made in the greenhouse using hand pollination in isolation under 18 hr. light and 6 hr. dark. About 384 F1 seeds were harvested, scarified, inoculated with rhizobium strain N-dure (INTX Microbials LLC, *Sinorhizobium meliloti* and *Rhizobium leguminosarum*), and grown in the greenhouse. In order to confirm the true hybrids, 24 simple sequence repeat (SSR) markers were screened for polymorphism between the parents. These markers were developed from *M. truncatula* [33] and were previously used to genotype tetraploid alfalfa [34]. From the set of 24 SSR markers, three markers with the strongest

amplification and highest polymorphic index between the two parents were used to genotype the F1 progeny. Two hundred true F1 hybrids were retained, but sufficient numbers of clones for the target locations and replications were obtained only from 184 hybrids. Twenty-four clones per entry were generated through stem cuttings and propagated in the field.

The two parents, 184 F1 progenies, 11 standard check cultivars for FD, and six checks for winter survival [35] were planted at two locations in Georgia. The first was the J. Phil Campbell Sr. Research and Education Center (JPC) in Watkinsville (33°52'17.8"N 83°27'05.5"W) and the other was the Georgia Mountain Research and Education Center at Blairsville (BVL) (34°50'21.4"N 83°55'20.5"W). The BVL location experiences frequently harsh winters and therefore is considered an ideal location to test alfalfa WH and persistence under cold stress. The average annual precipitation in the BVL location is 55.9 in, the average high temperature in July is 29 °C, and the lowest temperature in January is -4 °C. At the Watkinsville location, the average annual precipitation is 48 in, the highest temperature in July is 32.2 °C while the lowest temperature in January is 0 °C. The experimental design at each location was a randomized complete block, with three replications, where four clones from each progeny were planted in a single row plot. Plants were spaced 45 cm within each row, and the rows were spaced 90 cm from each other. Irrigation, fertilization and weed control were applied as necessary.

Genotyping-by-sequencing (GBS) and marker discovery

Single nucleotide polymorphisms (SNPs) markers were identified using genotyping-by-sequencing of the parents and progeny. DNA of each progeny and parents was extracted using CTAB method with some modifications [36]. Alfalfa tissue was collected in 50-ml tubes, freeze dried for 48 hr, and groynd using 6-8 zinc-plated copper balls in a Genogrinder (SPEX SamplePrep 2010 Geno/Grinder®) for 6 minutes at 1600 rpm. Then, 150 mg of the powder was

transferred to 2.0 ml tubes, 900 µl of CTAB buffer was added, vortexed, and the mixture was incubated at 65 ºC for one hour. Nine hundred µl of phenol:chlorofom:isoamyl alcohol (PCI) mix (25:24:1) with pH 5.0 was added to each tube, incubated for 15 minutes and centrifuged at 12500 rpm for 15 min and the clear supernatants were pipetted to new 2.0 ml tubes. Equal volume of chloroform:isoamyl alcohol (CIA) mix (24:1) was added to each tube, mixed gently and centrifuged at 12,500 rpm for 15 minutes. The aqueous upper phase was pipetted into 2.0 ml tube. About 0.6 volumes of chilled isopropanol was added to the tubes and left for 10 minutes, centrifuged at 13,000 rpm for 20 minutes. The DNA pellet was washed using 500 µl 70% ethanol, centrifuged at 7,500 rpm for 5 minutes. The supernatant was discarded, and the DNA pellet was air dried for 1-2 hours under the airflow. Then, 100 µl of sterile 10 mM Tris-HCl (pH 8.0) was added and incubated at 4 ºC for overnight.

The DNA solution was treated with 4 µl (10 mg/ml) of RNase A, followed by 5.0 µl (20 mg/ml) proteinase K, and incubated at 37 ºC in a water bath for 30 minutes after each addition. Sterile Millipore grade H_2O was added (400 µl) and treated with PCI and CIA as described earlier. The supernatant was pipetted into 1.5 ml tubes and 1/10 volume of 3M Na-acetate pH 5.2 (stored at 4 ºC) and 2.5 volumes of absolute ethanol was added, left for 10 minutes and centrifuged for 20 minutes at 13,000 rpm. The supernatant was discarded, and the pellet was washed with 70% ethanol, then air dried. The DNA was dissolved into 50-100 µl of sterile 10 mM Tris-Cl (pH 8.0). High quality DNA was ensured by quantification in Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA) and running the DNA samples in 1% agarose gel.

Two GBS libraries were constructed for 184 F1 progenies and the 2 parents. Both libraries were 96-plex including 92 F1 progenies and 2 replications of each parent. The barcode

adapters, common adapters, and two PCR primers were ordered from Integrated DNA Technologies (Coralville, IA, USA). The library was constructed using the protocol described in [28]. The DNA samples were digested with a methylation sensitive enzyme *ApeKI* and both common as well as barcode adapters were ligated. The step was followed by pooling the libraries (multiplexing) and cleaning up with Qiagen PCR (Qiagen, Germantown, MD) cleanup kit using the protocol provided with the kit. Moreover, the steps were followed by simple PCR using Kapa Library Amplification Readymix (Kapa Biosystems, Wilmington, MA) and two PCR primers. Finally, PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germantown, MD). Both libraries were submitted to Georgia Genomics and Bioinformatics Core (GGBC), UGA, for removing short fragments by solid phase reversible immobilization (SPRI), cleanup, and sequencing. Sequencing was performed on an Illumina Next Seq (150 Cycles) 75 PE High Output flow cell with four lanes. The raw sequence data was processed using two pipelines; GBS SNP Calling Reference Optional Pipeline (GBS-SNP-CROP) version 2.0 [37] and Tassel 3.0 Universal Network Enabled Analysis Kit (UNEAK) pipelines [38] for de novo SNP discovery. These bioinformatics computational steps were performed on in the Unix platform 'Zcluster' at the Georgia Advanced Computing Resource Center (GACRC), UGA.

The GBS-SNP-CROP was a useful tool for de novo SNP calling. The raw reads were parsed and trimmed for quality using Trimmomatic software version 0.36 [39]. The trimmed reads were demultiplexed producing high-quality reads for each genotype. The GBS specific mock reference was generated from parsed high-quality reads. The processed reads were mapped to generate standard alignment files using BWA-mem [40] and SAMtool version 1.3.1 [41]. Subsequently the SNP master matrix was produced, followed by SNP and genotype calling. The

raw sequence data was deposited at the NCBI SRA website under the accession number SRP150116 and it can be accessed at https://www.ncbi.nlm.nih.gov/sra/SRP150116.

Similarly, UNEAK pipeline was used to process the high quality R1 reads of pair-end data. In UNEAK, the raw R1 reads were filtered, de-multiplexed and trimmed to 64 bp. Similar reads were grouped as a tag, where tags with >10 reads were used for alignment in SNP calling. The parameters used to call and filter the homozygote alleles, heterozygote alleles, minor alleles etc. in UNEAK were as described [28]. The HapMap output files obtained from UNEAK were processed in Microsoft Excel for separating parental genotypes, removing missing data and testing for segregation ratios using chi-square.

Linkage map construction

Polymorphic SNPs unique to either 3010 (AB x AA) or CW 1010 (AA x AB) were screened as single dose allele (SDA) markers [28]. Parental genotypes that were heterozygous (AB) at any replication were considered heterozygous and parental genotype homozygous (AA) in all replications were considered homozygote. Markers that were missing in more than 30 % of the progeny were culled. The SDA markers obtained from both pipelines were added and input files were formatted as required by JoinMap 5.0 [32]. The SDA segregation ratio (1:1) was confirmed by chi-square tests ($p > 0.05$). The SNPs that were present in more than 30 % progenies but have segregation ratios other than 1:1 were considered in segregation distortion [28].

Both male and female SDA markers were loaded to JoinMap 5.0 separately. The markers were grouped using minimum independence LOD of 10. The grouped markers were mapped using regression mapping with a minimum LOD value of one, maximum recombination frequency of 0.40, and Kosambi mapping function. Linkage maps were generated using Map

Chart and the map files were exported. The tags of mapped markers from UNEAK pipeline were separated for each linkage group of both parents. Linkage groups were assigned chromosome numbers using the basic local alignment search tool (BLAST) for querying the consensus tags of SNPs with M. *truncatula* reference genome, *M. truncatula* V4.1 genome as described in [28].

Phenotypic data analysis and QTL mapping

Alfalfa dormancy data was collected as regrowth height after clipping in the fall and winter. In the fall, canopy height data was taken at four weeks after clipping the plants on 21st September according to NAAIC protocol [7]. The plant height data at the Watkinsville (JPC) and Blairsville (BVL) locations were taken in subsequent days. The mild winter of 2016/2017 in Georgia allowed taking an early winter and late winter regrowth data. FD was phenotyped in the parents and the pseudo-testcross progeny in fall 2015, fall 2016 and winter (2016/2017). We collected two winter data sets in the season (2016/2017). Because of the mild winter in the Southeastern U.S., it is possible to phenotype seasonal dormancy in field conditions later than northern environments. The regrowth height data was converted to FDR based on the regrowth of 11 standard checks according to NAAIC protocol. FDR of the progeny were assigned based on a regression equation derived from the relationship between standard dormancy ratings of the check cultivars and their regrowth height in each environment. The standard regression lines for each location were established using average dormancy values of three years. The equations were derived for all growing environments and seasons using the Proc Reg procedure in SAS 9.4 [42]

The dormancy phenotypic data consisted of two fall datasets (FD/2015 and FD/2016) for both locations, JPC and BVL, a winter data set collected in the first week of January (referred to as WD/2016 data set, and a second winter data set collected in the last week of February

(referred to as WD/2017 dataset). WH was evaluated on the F1 population and the two parents on a scale of 0-5 according to NAAIC protocol [43]. Visual scores of winter damage were recorded after each freezing occurrence in the winter months. In the case of mild winter, we visually scored plants once a month. Standard checks for winter survival were scored and photographed, and the images were used to guide in the scoring of F1 plants to minimize bias. The visual scores ranged from 1 to 5, where 1 indicates extremely winter-hardy genotypes and 5 indicates non-winter-hardy as described in NAAIC [43]. Phenotypic data for all traits was analyzed using SAS 9.4 [42]. The least square (LS) means for all genotypes across environments and within individual environments were estimated for each dataset using PROC GLM [18]. For each trait, a linear additive model was used to perform the analysis of variance (ANOVA) for randomized block design:

Trait value = genotype + environment + block (environment) + genotype*environment + Error

where the trait value refers to the trait phenotypic value estimated by combining the effects of genotype, environment, block, and genotype by environment interaction. The block (environment) was considered random [44]. The LS means of all traits for both parents were also obtained within each and across environments. The LS means of the progeny were used as trait value for QTL detection. Pearson correlation coefficients (r) were calculated for both FD and WH trait means within each environment.

QTLs were detected using composite interval mapping (CIM) algorithm on Windows QTL Cartographer version 2.5 (Statistical Genetics, NC State University). The model and parameters used for CIM analysis were as described [45]. We calculated trait-specific LOD

scores using 1000 permutations at genome wide statistical threshold ($P \leq 0.05$). A QTL was declared significant when the peak LOD value exceeded a conservative LOD threshold of 3.0. In the case of more than one peak, multiple QTLs were declared if LOD values between the peaks falls below 3.0 for more than one contiguous segment for at least one dataset analyzed [44].

The QTL detected in both parents for all traits were classified into two types, stable QTL and potential QTL. The QTL that were detected in more than one season, one environment, or across environments were considered stable QTL. QTL detected either in only one season or one environment were considered as potential QTL. The genomic positions of some major stable QTLs detected for each parental map were indicated on linkage maps using MapChart 2.3 [46]. The QTL detected for dormancy on the linkage map of the dormant parent, 3010, were given name as 'dorm'. Similarly, the QTL detected for non-dormant parent, CW 1010, were named as 'ndorm'. The QTL for WH that were detected in the winter-hardy parent 3010 were named as 'wh'. The QTL for WH trait detected in the cold susceptible parent (CW 1010) were labeled 'ws'. The QTL span was delimited using LOD-1 confidence interval and the QTL were considered identical when the 1-LOD support intervals for QTL overlaps as in previous report [44].

Results

GBS and SNPs discovery

A total of 100 Gb raw reads were generated using Illumina NextSeq High Output Flowcell (Illumina, Inc.) amounting to one billion usable paired-reads. Using the GBS-SNP-CROP pipeline for de novo SNP calling resulted in 4822 raw SNPs in the pseudo-testcross F1 population. There were 838 single dose allele (SDA) SNPs segregating in the maternal parent 3010 and 794 SNPs segregating in the paternal parent CW 1010. Among these, 423 SDA SNPs

from 3010 and 220 SNPs from CW 1010 were filtered as high-quality SDA after chi-square test ($\alpha = 0.05$) for the segregation ratio of 1:1 (AB: AA). The SNPs obtained from this pipeline were identified with suffix MRG referring 'Mock reference genome' followed by SNPs physical position with reference to MRG created using the reads of two parents.

Using the Tassel UNEAK pipeline, 500 million high-quality R1 reads were identified and processed using default parameter settings. A total of 65101 biallelic SNPs were identified. After filtering for missing data (<30%), 34122 (52.4%) SNPs were retained. Additionally, we removed 1625 loci with missing marker information in either parent retaining 32497 SNPs. Therefore, about 50% of the raw SNPs obtained from the UNEAK pipeline were filtered out in the initial screening because of missing data. Among the 32497 SNPs obtained from UNEAK, 4925 SNPs were single dose SNPs for 3010 parent and 2121 SNPs were identified as single dose SNPs for CW 1010 based on chi-square (α=0.05) test for segregation ratio (1:1).

Genetic mapping

After merging the GBS SDA-SNP obtained from both Tassel UNEAK and GBS-SNP-CROP pipelines for each parent, we generated a total of 5348 SNPs for the maternal parent 3010 and 2340 SDA for the paternal parent CW 1010 (File S1). Further screening of the SDA loci on JoinMap 5.0 [32] showed that three F1 individuals (ALF107, ALF255 and ALF302) did have several missing loci and were removed from further analysis leaving 181 progenies for mapping. Similarly, 26 loci from 3010 parent and 13 loci of CW 1010 parent were excluded from further analysis because they were identical. Consequently, 5322 SNPs from 3010 and 2327 SNPs from CW 1010 were used in genetic mapping.

SNPs from both parents were assembled into 32 linkage groups using LOD for independence of 12 or above in the 3010 parent and LOD of 10 or above in CW 1010. Based on

the SNP physical positions determined from BLAST analysis, each four linkage groups or haplotypes of each parent were assigned to a corresponding chromosome of *M. truncatula* as described in Figure 2.1 [28]. Since the majority of the SDA SNPs (92% SDA of 3010 and 90% SDA of CW 1010) were obtained from UNEAK, we only used the SNPs from this pipeline to query the physical locations of markers. The consensus sequence of tag pairs of all mapped SNPs of both parents used to query in nucleotide BLAST.

Thirty-two linkage groups of the maternal parent 3010 consisting of 1837 SDA SNPs were assembled in a linkage map spanning about 2788.4 cM with an average marker density of 1.5 cM/SNP (Table 2.1, Figure 2.S1). The number of SNPs per linkage group in 3010 parent ranged from 10 to 116. Most of the linkage groups ranged in length from 50 to 100 cM (Table 2.1, Figure 2.S1). Marker density of the individual LG varied from 0.9 to 5.3 cM/SNP.

The 32 linkage groups of CW 1010 spanned 2127.5 cM with 1377 mapped markers (Table 2.1, Figure 2.S2). The average marker density was 1.5 cM/SNP. The number of SNPs mapped on CW 1010 linkage groups varied from 7 to 139 (Table 2.1, Figure 2.S2). Most of the CW 1010 LG had genetic lengths of 40 to 90 cM. The shortest LG (3D) was 26.7 cM and the longest LG (4B) was 121.9 cM. The individual group marker density in CW 1010 linkage map varied between 0.2 to 6.6 cM/SNP (Table 2.1).

BLAST analysis showed that alfalfa genetic loci mapped in this study were syntenic with *M. truncatula* reference genome (Figure 2.1 and 2.2). From 1837 SNPs mapped in the 3010 parent, 967 (53%) SNPs were aligned to the *Medicago* reference genome with 84-100 % identity. On *Medicago* reference genome, 3010 SDA SNPs were aligned within range of 3.3 Kb to 56.4 Mb. The cut-off value used in BLAST analysis ranged from 2.06 E^{-06} to $2E^{-26}$. Similarly, 741 (53%) SNPs from the parent CW 1010 exhibited similarity with the *M. truncatula* genome with

sequence identity of 85 to 100% using the same cut-off value as for 3010 SNPs. CW 1010 SNPs and *M. truncatula* genome similarity were obtained within a range of 0.5 Kb to 56.3 MB.

Dot plot maps constructed for each parent using mapped SNPs syntenic to *M. truncatula* clearly displayed the grouping of markers on the 32 LG groups to the corresponding eight *Medicago truncatula* chromosomes (Figure 2.1 and 2.2) [28]. In the female parent 3010, a translocation of a segment of chromosome four into eight was observed in all four homologs of chromosome eight. Three homologs (4B, 4C, 4D) of chromosome four also possessed segment from chromosome eight, indicating the reciprocal translocation between chromosomes four and eight (Figure 2.1) [28]. Such translocation was also observed for parent CW 1010, more clearly on haplotypes 4B, 4D, 8A, 8B and 8D (Figure 2.2). Several other minor genome rearrangement events such as inversions and other translocations were present in several haplotypes in the maps of both parents. However, this study is focused on marker- trait association rather than structural analysis of the alfalfa genome.

Phenotypic evaluation and correlation between traits

Eight regression equations were generated to estimate FD of the mapping population at two locations in fall 2015 (FD/2015), fall 2016 (FD/2016), winter 2016 (WD/2016) and winter 2017 (WD/2017). The regression models suggested that the relationship between standard FDR and the canopy regrowth height of alfalfa checks was strong and positive. The regression coefficients (R^2) ranged from 0.37 for fall 2015 at BVL to 0.73 of winter 2016 dormancy of JPC environment with six out of the eight regression models having a coefficient of determinations $R^2 > 0.50$. The regression coefficients of the winter rating were higher than fall ratings at both sites.

There were significant differences between the genotypes ($P \leq 0.01$) and significant G x E for FD ratings in most dates except for FD/2015 data. Because of the significant G x E, the LS means for each trait were estimated separately for each location (Table 2.2). The R^2 values for each trait, derived from the ANOVA, varied from 0.59 to 0.87 indicating a good fit of the data to the respective linear model for individual tests (Table 2.2). Dormancy measured in winters (WD/2016 and WD/2017) were highly correlated to each other than the dormancy measured in fall (FD/2015 and FD/2016) in JPC environment (Table 2.3). The FD trait for winter 2017 rating showed the highest R^2 (0.87) and fall 2016 exhibited the least R^2 (0.59). The LS means estimated for traits and parents revealed the presence of transgressive segregation on both sides of the parents for both FD and WH traits (Table 2.2). Some past studies also reported the presence of transgressive segregation in alfalfa pseudo-testcross progeny for such traits [18].

There were significant differences between the genotypes ($P \leq 0.01$) for WH and significant G x E. Because of the significant G x E, the LS means were estimated for each location in addition to across locations (Table 2.2). The R^2 values for WH, varied from 0.63 to 0.79 indicating a good fit of the data to the respective linear model for individual tests (Table 2.2). The LS means estimated for F1 progenies and parents revealed the presence of transgressive segregants for WH on both sides of the parents (Table 2.2).

Pearson's correlation coefficient using trait means showed moderate degrees of correlation between all traits at both locations (Table 2.3 and 2.4). Overall, there were stronger positive correlations between dormancy and WH when dormancy was assessed in winter compared to fall assessment (Figure 2.3, Tables 3, 4). In the JPC environment, the coefficient of correlations between dormancy rating and WH ranged from 0.12 to 0.57 when dormancy was assessed in the fall, while it ranged from 0.22 to 0.85 when dormancy was assessed in winter

(Table 2.3). The same trend was observed in the BVL location. The coefficient of correlations between dormancy rating and WH ranged from 0.16 to 0.50 when dormancy was assessed in the fall, while it ranged from 0.22 to 0.57 when dormancy was assessed in winter (Table 2.4).

QTL mapping of FD and WH

Within the 32 homologs of the 8 alfalfa chromosomes, we detected 45 significant ($P < 0.05$) QTLs for FD and 35 QTLs for WH on both male and female linkage maps (Table 2.5-2.8). Most of the QTLs detected using phenotypic data across environments matched QTLs detected for individual environments with slight variation in their LOD magnitude and interval. Seven QTLs for dormancy and three QTLs for WH detected across environments were exclusively different from QTLs detected for individual environments indicating a potential effect of G x E on trait values (Table 2.8).

Fall dormancy (FD)

Seven stable QTL for FD were identified in the dormant parent 3010. These QTLs were consistently and repeatedly detected across data sets within overlapping 1-LOD support intervals (Table 2.5, Figure 2.4). The seven dormancy QTL for 3010 (dorm1, dorm2, …..., dorm7) were detected on homologs 1A, 3A, 4C, 7A and 7B. Another 21 potential QTLs (dorm8, dorm9, ...…, dorm28) were detected in various homologs of 3010 chromosomes: 1, 2, 3, 4, 5, 6 and 7 (Tables 2.5, 2.7 and 2.8). Five of the seven stable QTLs were detected also across environments. The most important dormancy QTL (dorm1) for 3010 parent ($R^2 = 0.16$) was detected on homolog 1A and was located at 90.6 – 92.9 cM. The same homolog harbors another QTL (dorm2) with a LOD value of 6.2 and a peak at the interval 98.2 – 104 cM (Table 2.5, Figure 2.4). Besides these two stable QTLs for 3010 parent, other potential QTLs (dorm 8, dorm 12, dorm 27 and dorm 28)

were detected on homologs of 3010 chromosome 1, suggesting that this chromosome is important for the dormancy trait.

Further, dorm1 and dorm2 QTLs of 3010 parent mapped on homolog 1A were located within a similar genomic location of alfalfa fall height QTLs (92a and 104a) in the WISFAL-6 cultivar reported previously [18]. Li et al. 2015 mapped fall height on eight alfalfa linkage groups assigned using eight *M. truncatula* chromosomes. Unlike 3010 QTLs dorm1 and dorm2 detected in our study, WISFAl-6 dormancy QTL of LG1 had positive effect on trait value because the source parent WISFAL-6 had higher FD levels [18].

Another two stable dormancy QTLs from 3010 were detected on chromosome 7A (R^2 = 0.07-0.11). Homologs of this chromosome also harbor QTL dorm3, dorm5, dorm6, and dorm13 (Table 2.5 and 2.7). The QTL dorm6 also falls within similar genomic regions of a fall height QTL in LG 7 as reported previously [18]. Two other potential QTL on homologs of this chromosome at LOD = 3.1 include dorm21 and dorm22 that were located on two homologs (7D and 7C) of 3010 chromosome7. Further, the homologs of 3010 chromosomes: 3, 4 5, 6, and 2 also harbored significant ($P < 0.05$) QTLs for dormancy (Table 2.5 and 2.7). All dormancy QTLs detected on 3010 parent had negative effects on trait value since the parent was a dormant type.

In the CW 1010 parent, 11 stable QTLs and six potential QTLs for FD were detected. All the stable QTLs for CW 1010 were detected on homologs of the chromosomes 1, 7, 5, and 8 (Table 2.5). The CW 1010 chromosome 8 exhibited a broader QTL peak extending from ~44 to ~ 66 cM. However, there is the possibility of presence of more than one QTL in the region because of decreasing LOD value between multiple QTL peaks. Therefore, we reported three different stable QTLs for this region to ensure the accuracy of QTL and corresponding phenotypic values of markers in the region. A past study [18] also reported a QTL (46a)

positively affecting fall plant height in the same genomic region (40- 56 cM) of LG 8 of the alfalfa cultivar ABI408 providing more supportive evidence for this QTL. The QTL (ndorm1) from CW 1010 with positive effect on the trait value (R^2 =0.13) was detected on homolog 8D at the interval 44.6 – 46.3 cM (Table 2.5). Other stable QTLs for dormancy detected from CW 1010 parent including ndorm2, ndorm3, ndorm4 and ndorm6, shared common genomic regions with QTLs for fall height reported previously [18]. However, two CW 1010 dormancy QTLs, ndorm3 and nodorm6, had contrasting effects in trait value than previously reported QTLs of the corresponding linkage groups. Of 17 total CW 1010 dormancy QTLs, 16 QTLs had additive effect in favor of trait value and one potential QTL (ndorm13) had negative effect on trait value (Table 2.7).

Although we detected dormancy QTLs for both 3010 and CW 1010 parents in most of the datasets, a higher number of stable QTLs were repeatedly detected using winter dormancy data compared to fall data of all environments (Table 2.5, 2.7 and 2.4). For the 3010 parent, only two stable QTLs were observed for each 2015 and 2016 fall datasets of BVL location, while two and four stable QTLs were detected for JPC winter datasets WD/2016 and WD/2017, respectively (Table 2.5). Only two stable dormancy QTLs for 3010 parent (dorm1 and dorm4) were repeatedly detected in fall. However, four stable dormancy QTLs (dorm2, dorm3, dorm5 and dorm6) were repeatedly detected in more than one winter data (Table 2.5). For CW 1010 parent, out of eleven stable dormancy QTLs, only six stable QTLs were identified for all FD data of both locations, and nine stable QTLs were detected for winter datasets of both locations (Table 2.5). Five and three potential QTLs were detected only in cross environments analysis for both parents 3010 and CW 1010 indicating the presence of G x E (Table 2.5). There was also

slight shift of QTL peaks identified in across environments compare to those QTL identified in individual environment data sets.

Winter hardiness (WH)

Eight stable and eleven potential QTLs were identified from the 3010 parent for WH trait (Table 2.6 - 2.8). The stable QTLs (wh1, wh2, ……, wh8) were detected on homologs of chromosomes: 1, 3, 4, 7 and 8, and twelve potential QTLs (wh9, wh10, ……, wh20) were detected on homologs of chromosomes: 2, 3, 4, 7 and 8. The QTL wh1 on homolog 1A (position 90 – 93.2 cM) has the largest R^2 (0.13) followed by QTL wh2 on homolog 7A (0.12) and wh3 on homolog 1A (0.11) (Table 2.6). The wh1 QTL was located in the same genomic region of previously identified QTL (100a) in WISFAL-6 alfalfa, but with opposite effect [18]. Similarly, other two potential QTLs, wh10 on homolog 7C and wh16 of homolog 2C were also detected within similar genomic locations of previously identified QTLs for winter injury for ABI408 (LG7, 109a) and WISFAL-6 cultivar (LG2, 36b), respectively [18]. With the exceptions of a stable QTL (wh4) on homolog 8A and one potential QTL (wh9) on homolog 7C, which had negative effects (-) on WH, all other WH QTLs detected on 3010 possessed positive effects (+) on WH (Tables 6-8).

Sixteen (nine stable and seven potential) QTLs for WH were detected for the winter susceptible parent CW 1010 and were coded as (ws1, ws2, …, ws16) (Table 2.6 and 2.7). Major stable QTLs for WH in CW 1010 were detected on homologs of chromosomes 1, 7 and 8. The QTL ws1 had the highest R^2 (0.14) followed by ws2, ws3, and ws4 (R^2= 0.10). However, contrary to ws1 and ws2, the ws3 and ws4 QTLs had positive effects (+) on WH (Table 2.6). The three stable QTLs, detected for WH trait on homolog 8D, appeared in a single span for JPC data. However, for BVL and across environments, the QTL on 8D was separated into three different

QTLs and were reported as such. Of the total 16 WH QTLs in CW 1010, ws12 on homolog 5A and ws16 on homolog 6A were detected within similar genomic regions reported previously for winter injury in the cultivars WISFAL-6 and ABI408 [18]. Most of the WH QTLs for CW 1010 have negative effects (-) on the trait except QTL ws3, ws4, ws9, ws11 and ws15 (Table 2.6 and 2.7).

Association between dormancy and winter hardiness

Among the seven stable dormancy QTLs detected in the 3010 the dorm1 and dorm2 on homolog 1A shared the same genomic location as WH stable QTLs wh1 and wh3, respectively (Figure 2.4). Similarly, the dormancy QTL dorm3 overlapped with WH QTL wh2 on 7A with less than 1 cM shift (Figure 2.4). Another stable QTL dorm6 also shared the same genomic location with a potential winter hardiness QTL wh20 on 7A. Three stable QTLs (dorm4, dorm5, and dorm7) in the 3010 parent were unique and located on different chromosomes than winter hardiness. Of the 21 potential dormancy QTLs in the 3010 parent, except dorm14 and dorm24, other 19 were also located in different genomic regions from the QTLs of WH (Table 2.5-2.8). Therefore, of the 28 dormancy QTLs detected in 3010 parent, 22 QTLs were located in separate genomic positions than the QTLs of WH indicating differences of two traits at the genomic level.

In the CW 1010 parent, the stable QTLs ndorm1 and winter hardiness QTL ws5 shared the same genomic location on homolog 8D (Figure 2.5). Likewise, the QTL ndorm2 and ws1 also reside on the same position on homolog 7C. Another stable dormancy QTL of CW 1010 ndorm8 shares genomic location with a potential QTL for winter hardiness ws13 on homolog 1B. Moreover, a stable dormancy QTL ndorm7 and a winter hardiness QTL ws8 were located at nearby positions on the homolog 8D (Figure 2.5). Similarly, ndorm10 and ws7 also shared partial genomic position on homolog 7B of CW 1010 (Figure 2.5). Other stable dormancy QTLs

from the parent CW 1010 such as ndorm3, ndorm4, ndorm5, ndorm6, ndorm9, and ndorm11 were located in separate genomic positions than those QTLs for WH. All potential QTL detected for CW 1010 for dormancy as well as for WH were also located in distinct genomic regions (Table 2.5-2.8). Therefore, of the 17 dormancy QTLs detected in CW 1010 parent, 12 QTLs were located in separate genomics regions than the QTLs for WH (Table 2.5-2.8).

Discussion

Segregation and phenotypic relationships between traits

The regrowth height and dormancy ratings of the NAAIC standard checks used in this study showed a better fit to the regression model in winter height data with R^2 up to 0.73 compared to the height data taken in fall ($R^2 \sim 0.50$) (Table 2.3 and 2.4). The fall data is collected around the third week of October according to NAAIC protocol. In southern environments, temperatures at this time of the year are still very favorable for active growth of alfalfa. Fall 2016 was a very unusual season in Georgia because of the historical drought in the region [47], which led to a very limited growth and erratic regrowth after clipping in both experimental sites. This could be the main reason that the two parents did not exhibit differences in their heights for this season (Table 2.2) and a few QTLs were detected based on the 2016 fall data. The positive R^2 for the regression of standard checks regrowth height data on their FDR for all seasons, suggests that that determining dormancy of alfalfa genotypes using regrowth height after clipping is a reliable approach.

The non-dormant parent CW 1010 exhibited slightly lower dormancy level (4.7 – 7.9), than it supposed to be in most of the years. The 3010 parent mostly exhibited the expected dormancy level between 2.3 to 6.4 (Table 2.2). Such fluctuations of estimated dormancy level from their standard dormancy are primarily due to environmental and seasonal variations. The

largest deviations from the standard dormancy ratings of the parents were observed in the data collected in the fall season, suggesting that rating alfalfa dormancy in the Southeastern U.S.A based on regrowth height after clipping in third week of September is not reliable. Previous reports also suggested that FD assignment should be done in sites where the cultivars are broadly adapted [7]. Dormancy assessment in winter months showed a better approximation of the expected values with less variation compared to the fall assessment. Winter assessed dormancy also showed a better repeatability in both locations as suggested by the high correlation between WD/2016 and WD/2017 ($r = 0.92$, $p < 0.01$) (Table 2.3 and 2.4). The LS means of dormancy ratings of the F1 progeny varied from 1.2 to 10.6 and showed transgressive segregation around the parental values (Table 2.2).

WH rating scores for 3010 parent varied from 1.4 to 1.5 across locations, which is within the range of the known score 2 for this cultivar. For the non-winter-hardy parent CW 1010, the scores varied between 2.3 to 4.1 across locations (Table 2.2). The F1 progeny also varied in their WH level with the largest differences observed in 2017 (WH/2017) at BVL location. Transgressive segregants were observed for both dormancy and WH similar to previous alfalfa reports [18]. This suggests the presence of complementary alleles for both traits in each of the parents [18]. The moderate positive correlations (r) observed between dormancy and WH irrespective of the time of assessment is a clear indication of the weak association between the two traits (Table 2.3 and 2.4).

Genetic linkage map

Genetic maps are important tools for genetic analysis of quantitative traits through QTL and comparative mapping. They are also useful for genome assembly and marker development for MAS [28,48]. In alfalfa, genomic resources are very scarce and even though a limited

number of genetic maps were published, there is no consensus map to date. Alfalfa linkage maps reported so far have variable sizes and marker density depending on the type of mapping population, maker types, and software used. Most studies reported tetraploid alfalfa maps either for 8 linkage groups or for 32 linkage groups for each parent [18,49]. An early reported tetraploid alfalfa linkage map covered only 443 cM for seven linkage groups [50]. Julier et al. (2003) constructed alfalfa genetic maps using SSR and AFLP for eight linkage groups containing four homologs, where the parental maps spanned 2649 and 3045 cM [49]. The authors argued that their alfalfa genetic maps were close to saturation and exhibited high level of collinearity with other maps of alfalfa and *M. truncatula* [49]. However, the genetic maps they constructed were relatively less dense (7 – 9 cM/marker). Musial et al. (2007) reported alfalfa linkage maps using a backcross (BC) population, where the eight linkage groups spanned 794.1 cM with 3.9 cM/marker [51]. Li et al. 2015 constructed linkage maps of WISFAL-6 and ABI408 with respective lengths of 898 cM and 845 cM for 8 linkage groups [18]. The linkage map constructed in this study is the second high-density genetic map, published so far, after the alfalfa linkage maps described by Li et al. (2014b) for two alfalfa genotypes DM3 and DM5. Moreover, our linkage maps for both 3010 and CW 1010 parents have almost similar average marker density (~ 1.5 cM/SDA-SNP) to the linkage map of parent DM3 [28]. Our linkage maps also exhibited high levels of synteny with the *M. truncatula* genome as in [49,28]. The total length of the 3010 map (2788.5 cM) was slightly higher than the CW 1010 map (2127.55 cM). Such differences in parental linkage maps were also observed in previous alfalfa genetic linkage maps [49]. The difference might be due to more SDA markers segregating in 3010 parents (5348) compared to (2340) segregating for CW 1010. The higher number of markers in 3010 may be resulted because of the higher number of recombinations that lead to a longer linkage map. In *Brassica*

oleracea, the recombination frequency in female meiosis is higher than the male, which obviously generates more markers for the female linkage map and therefore a longer map than the male one [52]. However, in alfalfa there are no reports available regarding sex related differences in meiosis frequency. As we obtained a lower number of raw reads for CW 1010 parents than 3010 in either replications, we believe that these differences could result in a low number of markers for CW 1010. Furthermore, since GBS is a reduced representation approach, the number and quality of genotype calls may vary between individuals [53].

Mapping and QTL detection

Constructing linkage maps based on single dose markers (1:1) in outcrossing polyploid species and using the maps for linkage analysis of quantitative traits has been a common practice for decades [54]. The pseudo-testcross strategy uses the heterozygous markers for one parent and double null in other parents for mapping, which further uses inbred backcross configuration in mapping software [55,56]. This mapping strategy has been successfully used before in tree plants such as *Eucalyptus grandis* and *Eucalyptus urophylla* [57], *Pinus elliottii* and *P. caribaea* [58], and grass such as orchardgrass (*Dactylis glomerata* L.) [59]. This mapping strategy possesses however some drawbacks [60]. Dominant and additive effects on QTL are confounded and the effects of alleles that were substituted only from other parents are obtainable [61,62]. Since the parents of the pseudo-testcross population are heterozygous, the marker and QTL alleles may be in different states and linkage phases, which makes the strategy less powerful than the classical QTL analysis in inbred populations [62] in addition to mapping only a portion of markers [63]. Nevertheless, this strategy is still useful in detecting QTL, displaying the direction and magnitude of QTL effect and the position of QTL in species with complex genome such as polyploids.

In this study, we used the pseudo-testcross strategy with GBS SNPs in alfalfa for QTL mapping of dormancy and WH in alfalfa. A total of 45 QTLs associated with FD and 35 QTLs for WH were mapped on two alfalfa cultivars CW 1010 (male) and 3010 (female) genetic maps. Even though previous studies reported QTL mapping of dormancy in alfalfa, these maps were based on parents relatively close in dormancy and constructed with traditional markers [18,64,65]. Furthermore, with 11 dormancy classes and 6 classes of WH it is very unlikely to capture the majority of loci underlying these traits in a bi-parental population. The latest alfalfa QTL map reported QTLs for winter injury and FD, but with large QTL intervals (>10 cM) [18] leading the authors to suggest the need for further research to narrow down QTLs positions. The mapping population was also generated from a dormant × a semi-dormant and winter-hardy parents (WISFAL-6 x ABI408) which makes it difficult to capture the alleles underlying non-dormancy and cold susceptibility. In this study, the QTLs were detected within 1-LOD support interval with flanking and peak markers for all QTLs identified (Table 2.5-2.8). Some of the QTLs detected in this study were located in the same genomic locations as previous studies [18].

Because of the genotype by environment interactions, the QTLs for FD and WH were categorized into stable QTLs that were consistently expressed in more than one environment and potential QTLs that were detected just in an individual environment for one season or only across environments. Considering QTL×E in the analysis enhances the precision of QTL study since the multi-environment QTL test is more powerful in comparison to single-environment analysis [66]. Therefore, to verify the alfalfa WH and FD QTLs detected in our analysis validation studies need to be conducted in other environments using different alfalfa backgrounds.

Association between FD and WH

Phenotypic and genetic relationship between alfalfa FD and WH has been a matter of debate for a longtime. Earlier studies reported that alfalfa dormancy and WH are phenotypically correlated (r = 0.90) and most likely genetically associated leading breeders to use one trait as surrogate to select for the other [10,67]. Recent studies re-examining this relationship argued for weaker genetic linkages between the traits and suggested that improving one trait by selecting for the other may not be successful [10,18]. Other findings suggested that the relationship between WH and FD in alfalfa depends on the type of germplasm tested [10], implying that the two traits could be manipulated independently [68,10].

In this study, we observed moderate positive phenotypic correlations between WH and FD in the F1 pseudo-testcross population. The magnitude of correlation, however varied with the assessment time of FD. Dormancy measured in the fall after clipping on 21 September showed a weaker relation to WH than dormancy assessed in winter. Assessing regrowth height for FD in areas with warmer late autumn temperatures may not be reliable and should be delayed to early winter. Nevertheless, for reliable ratings of FD and WH, multi-years data are necessary [7].

The QTL analysis performed in this study revealed that more than 75 % (22/28) of the dormancy QTL detected for the 3010 parent did not share genomic regions with winter hardiness QTLs (Table 2.5-2.8). Similarly, for the CW 1010 parent, more than 70% (12/17) dormancy QTLs detected were localized in different genomic regions than winter hardiness QTLs. These results clearly suggest that the two traits are inherited separately and therefore can be genetically manipulated independently in breeding programs [18,10]. The dormancy QTLs (dorm1, dorm2; ndorm1, ndorm4) sharing common genomic regions with winter hardiness QTLs (wh3, ws2, ws5) in the two parents might have been the result of pseudo-linkage resulting from the

simultaneous long-term selection for the two traits. It is important to note that a pseudo-testcross population does not provide enough recombination to break apart closely linked loci. Previous QTL mapping work also found few overlapping QTLs for dormancy and winter injury suggesting genetic relation between the traits [18]. Since the two parents used in our study are more phenotypically divergent (FDR 2 for 3010 vs FDR 10 for CW 1010) in both dormancy and WH than any of the parents used in previous studies, the genetic linkage between the two traits is most likely tighter [68]. The QTLs detected in this study will be a valuable addition to the genomic resources for alfalfa breeding programs and to the understanding of the genetic basis of seasonal dormancy and winter hardiness. The segregating non-dormant genotypes with low winter injury generated in this study constitute a valuable germplasm resource to develop winter-hardy non-dormant cultivars.

Tables and Figures

Table 2.1. Distribution of SNP markers on 32 linkage groups of each of two alfalfa parents (CW 1010 and 3010). Number of markers, genetic length, and marker density for each homologs group are indicated. The homologs groups (A, B, C and D) were assigned randomly within each chromosome based on BLAST search result.

Chr[+]	Homologs group	CW 1010			3010		
		No. SNPs	Length cM	MD[¥]	No. SNPs	Length cM	MD[¥]
1	A	84	56.7	0.7	59	108.5	1.8
1	B	77	73.1	0.9	75	95.1	1.3
1	C	22	71.3	3.2	30	109.6	3.7
1	D	12	40.3	3.4	58	91.6	1.6
2	A	53	114.5	2.2	48	88.9	1.9
2	B	61	78.5	1.3	20	32.7	1.6
2	C	26	76.1	2.9	10	53.3	5.3
2	D	11	73.1	6.6	16	13.6	0.9
3	A	39	70.7	1.8	79	91.9	1.2
3	B	41	50.9	1.2	45	106.7	2.4
3	C	27	62.1	2.3	51	85.9	1.7
3	D	22	26.7	1.2	55	85.7	1.6
4	A	7	48.75	7.0	64	82.1	1.3
4	B	56	121.9	2.2	53	101.2	1.9
4	C	82	72.6	0.9	70	85.5	1.2
4	D	31	71.6	2.3	116	110.4	1.0
5	A	65	88.2	1.4	65	83.1	1.3

5	B	49	84.3	1.7	30	112.3	3.7
5	C	45	47.1	1.0	72	79.2	1.1
5	D	9	51.1	5.7	44	91.2	2.1
6	A	26	55.5	2.1	64	90.3	1.4
6	B	24	42.1	1.8	80	87.9	1.1
6	C	48	91.8	1.9	69	84.9	1.2
6	D	74	91.2	1.2	42	84.2	2.0
7	A	64	83.1	1.3	74	92.3	1.2
7	B	71	46.9	0.7	37	97.3	2.6
7	C	33	82.3	2.5	86	112.1	1.3
7	D	11	42.7	3.9	82	75.9	0.9
8	A	139	28.1	0.2	44	97.9	2.2
8	B	7	46.5	6.6	76	92.1	1.2
8	C	35	66.1	1.9	65	77.8	1.2
8	D	26	71.7	2.8	58	87.2	1.5
Total		1377	2127.55	1.5	1837	2788.4	1.5

Chr[+]= Chromosomes; MD[¥]= Marker Density (cM/SNPs)

Table 2.2. Phenotypic means of F1 progeny and parents for FDR and WH scores. Coefficient of determination (R^2) are indicated for each data set.

Trait/Year	Location	F1 phenotype range (LS Means)	LS means (3010)	LS means (CW 1010)	(ANOVA, F1) R^2
FD/2015	JPC	2.3 – 9.0	6.4	7.5	0.73
FD/2016	JPC	1.9 – 7.1	4.6	4.7	0.59
WD/2016	JPC	2.0 – 7.3	2.3	5.7	0.82
WD/2017	JPC	1.2 – 8.8	2.7	5.3	0.87
FD/2015	BVL	2.2 – 9.1	5.7	6.6	0.71
FD/2016	BVL	2.89 – 10.6	4.5	6.5	0.61
WD/2016	BVL	2.5 – 8.4	4.5	7.9	0.77
WD/2017	BVL	1.6 – 9.4	4.8	7.5	0.73
FD/2015	JPC & BVL	2.6 – 8.5	6.1	7.0	0.67
FD/2016	JPC & BVL	3.4 – 8.0	4.5	5.6	0.62
WD/2016	JPC & BVL	2.3 – 7.6	3.8	6.8	0.80
WD/2017	JPC & BVL	1.6 – 8.3	3.7	6.5	0.80
WH/2015	JPC	1 – 3.2	1	2	0.70
WH/2016	JPC	1.2 – 5.0	2.5	4.2	0.79
WH/2017	JPC	1 – 4	1	2.5	0.79
WH/2015	BVL	1 – 5	2	2.7	0. 71
WH/2016	BVL	1 – 4	1.3	4	0.63
WH/2017	BVL	1 – 4.9	1.7	4.5	0.70
WH/2015	JPC & BVL	1 – 3.4	1.5	2.3	0.79
WH/2016	JPC & BVL	1.4 – 4.1	1.8	4.1	0.78
WH/2017	JPC & BVL	1 – 4.3	1.4	3.5	0.78

FD = dormancy assessed in fall; WD = dormancy assessed in winter; WH = winter hardiness

Table 2.3. Phenotypic correlations (r) among traits based on data collected for Watkinsville (JPC) environment on a pseudo-testcross F1 population (3010 × CW 1010). Dormancy was assessed twice in the fall (FD/2015 and FD/2016) and twice in the winter (WD/2016 and WD/2017). The WH data was collected in three consecutive winters (WH/2015, WH/2016 and WH/2017).

	FD/2015	FD/2016	WD/2016	WD/2017	WH/2015	WH/2016	WH/2017
FD/2015		0.50 **	0.62 **	0.60 **	0.39 **	0.52 **	0.57 **
FD/2016			0.39 **	0.43 **	0.12 NS	0.31 **	0.50 **
WD/2016				0.92 **	0.22 **	0.65 **	0.80 **
WD/2017					0.23 **	0.71 **	0.85 **
WH/2015						0.16 *	0.10 NS
WH/2016							0.68 **
WH/2017							

* P < 0.05, **, P < 0.01, NSnon-signifiant

Table 2.4. Phenotypic correlations among traits based on data collected at the BVL location on a pseudo-testcross F1 population (3010 × CW 1010). Dormancy was assessed twice in the fall (FD/2015 and FD/2016) and twice in the winter (WD/2016 and WD/2017). Winter hardiness (WH) data were collected in three consecutive winters (WH/2015, WH/2016 and WH/2017).

	FD/2015	FD/2016	WD/2016	WD/2017	WH/2015	WH/2016	WH/2017
FD/2015		0.42**	0.6**	0.58**	0.16*	0.16*	0.33**
FD/2016			0.6**	0.64**	0.25**	0.43**	0.50**
WD/2016				0.92**	0.24**	0.27**	0.57**
WD/2017					0.22**	0.25**	0.51**
WH/2015						0.34**	0.46**
WH/2016							0.54**
WH/2017							

* P < 0.05, ** P < 0.01, ^{NS}non-signifiant

Table 2.5. Stable QTLs for alfalfa FD detected in an F1 (3010 × CW 1010) pseudo-testcross population based on phenotypic data assessed in fall and winter at two locations. Seven QTLs for 3010 and eleven QTLs for CW 1010 were mapped on respective genetic linkage maps for phenotypic datasets of more than one environment and/or year. The symbol with bracket in the column 'year/location' indicates the source dataset from which the other parameters in the same row were pulled.

Parent	QTL Code	Chr.	Year/Location	Peak Markers	Peak LOD	R^2	Allele Dir.	LSI (cM)	Flanking Markers
3010	dorm1	1A	(π1), π4, β2, $1, $2	TP995	10.9	0.16	(-)	90.6 - 92.9	TP995 - TP78651
3010	dorm2	1A	π1, π3, (π4), β3, β4, $1, $4	TP86274	6.2	0.11	(-)	98.2 - 104	TP65855 - TP86274
3010	dorm3	7A	π1, (β3), β4, $1	TP24733	6.2	0.11	(-)	36.9 - 38.8	TP55743 - TP34483
3010	dorm4	4C	(π1), β1, $1	TP56893	7.5	0.11	(-)	58.6 - 61.0	TP55689 - TP56893
3010	dorm5	7B	(π4), β3	TP31689	4.1	0.08	(-)	34.6 - 48.7	TP31689 - TP33803
3010	dorm6	7A	(π1) π3, π4, β3, β4, $1, $3	TP69889	5.5	0.07	(-)	47.3 - 52	TP59349 - TP71458
3010	dorm7	3A	β1, β2, (β3)	TP32327	3.8	0.06	(-)	25.8 - 26.3	TP3895 - TP54529
CW 1010	ndorm1	8D	(π1) π3, π4, β1, $1	TP2543	9.9	0.13	(+)	44.6 - 46.3	TP2543 - TP88682
CW 1010	ndorm2	7C	π3, (π4), β3	TP38417	9.0	0.12	(+)	46.9 - 51.1	TP38417 - TP54614
CW 1010	ndorm3	5B	(β1), $1	MRG_32692305	4.4	0.08	(+)	15.2 - 17.9	TP79886 - MRG_32692305
CW 1010	ndorm4	8D	π3, (π4), β1, $3	TP24024	6.1	0.07	(+)	53.7 - 54.8	TP24024 - TP25406

CW 1010	ndorm5	1B	π1, (β4), $4	TP57411	5.1	0.07	(+)	14.2 - 15.3	TP63551 - TP32288
CW 1010	ndorm6	5B	(π1), β2	TP26770	5.5	0.06	(+)	48 - 49.5	MRG_37364973 - TP26770
CW 1010	ndorm7	8D	π3, (π4)	TP67491	3.9	0.06	(+)	63.9 - 65.4	TP67491 - TP71707
CW 1010	ndorm8	1B	π1, π3, β1, (β4), $4	TP41786	3.2	0.05	(+)	21.1 - 23.7	TP83000 - TP7086
CW 1010	ndorm9	1B	(π3), π4, β3	TP52371	4.2	0.05	(+)	31.9 - 33.8	TP35547 - TP23336
CW 1010	ndorm10	7B	π3, (π4)	TP14107	3.8	0.04	(+)	23.3 - 24.2	TP14107 - TP9019
CW 1010	ndorm11	7B	π3, (π4)	TP7325	3.1	0.03	(+)	11.7 - 12.7	TP32866 - TP42483

JPC environment dormancy data for the period:

π1 = 2015, fall; π2 = 2016, fall; π3 = 2016, winter; π4 = 2017, winter

BVL environment dormancy data for the period:

β1 = 2015, fall; β2 = 2016, fall; β3 = 2016, winter; β4 = 2017, winter

Across environment dormancy for the period:

$1 = 2015, fall; $2 = 2016, fall; $3 = 2016, winter; $4 = 2017, winter

Other abbreviations:

Chr. = Chromosome; Dir. = Direction; LSI = 1-LOD support interval in cM unit

Table 2.6. Stable QTLs for alfalfa WH identified in an F1 (3010 × CW 1010) pseudo-testcross population based on phenotypic data assessed in three consecutive winters at two locations. Eight QTLs from 3010 and 9 QTLs from CW 1010 were mapped on respective genetic linkage maps using phenotypic datasets of more than one environment and/or year. The symbol with bracket in the column 'year/location' indicates the source dataset from which the other parameters in the same row were generated.

Parent	QTL code	Chr.	Year/Location	Peak Marker	Peak LOD	R^2	Allele dir.	LSI (cM)	Flanking Markers
3010	wh1	1A	(λ2), λ4, ϕ3	TP995	7.1	0.13	(+)	90.8 - 93.2	TP995 - TP6492
3010	wh2	7A	(λ3), ψ1, ϕ1	TP24733	7.1	0.12	(+)	37.5 - 39.0	TP55743 - TP34483
3010	wh3	1A	(λ2), ϕ3	TP65855	5.4	0.11	(+)	98.2 - 104.5	TP65855 - TP86274
3010	wh4	8A	(ψ2), ϕ2	TP10810	5.1	0.09	(-)	74.1 - 74.8	TP58070 - TP10810
3010	wh5	3A	(ψ3), ϕ3	TP71671	5.1	0.09	(+)	47.6 - 50.1	TP52425 - TP67563
3010	wh6	1C	(λ1), ψ1, ϕ1	TP37162	4.1	0.07	(+)	96.3 - 99	TP37162 - TP57104
3010	wh7	7A	(λ1), λ3, ϕ1	TP58371	3.2	0.07	(+)	26.4 - 29.3	TP58371 - TP34795
3010	wh8	4C	(λ2), ψ1, ϕ1	TP2323	3.5	0.06	(+)	27.1 - 31.1	TP6532 - TP4218
CW 1010	ws1	7C	λ2, (λ3), ψ1	TP54614	9.8	0.14	(-)	48.4 - 51.4	TP38417 - TP54614
CW 1010	ws2	8D	(λ3), ψ3, ϕ3	TP52817	7.5	0.10	(-)	40.8 - 43.6	TP52817 - TP46951
CW 1010	ws3	7A	(ψ3), ϕ2	TP71946	5.7	0.10	(+)	8.6 - 17.6	TP78230 - TP71946
CW 1010	ws4	7A	(Ψ2), ϕ2	TP81779	5.3	0.10	(+)	31 - 32.1	TP16325 - TP70376

CW 1010	ws5	8D	(λ2), ϕ3	TP2543	5.2	0.08	(-)	44.6 - 45.8	TP2543 - TP6748
CW 1010	ws6	7B	(λ3), ψ1, ϕ1	TP87913	5.6	0.07	(-)	35.9 - 37	TP87913 - TP85708
CW 1010	ws7	7B	(λ3), ψ1, ϕ3	MRG_41805356	4.9	0.07	(-)	24.5 - 25.3	TP49165 - TP74211
CW 1010	ws8	8D	(λ1), λ2	TP8426	3.3	0.06	(-)	58.6 - 63.1	TP69982 - MRG_7512818
CW 1010	ws9	1A	(λ3), ϕ3	TP40020	3.4	0.04	(+)	47.2 - 48	TP60690 - TP40020

JPC environment winter hardiness data for the period:

λ1 = 2015; λ2 = 2016; λ3 = 2017

BVL environment winter hardiness data for the period:

ψ1= 2015; ψ2 = 2016; ψ3 = 2017

Across environment winter hardiness data for the period:

ϕ1 = 2015; ϕ2 = 2016; ϕ3 = 2017

Table 2.7. Potential QTLs for dormancy and WH identified in an F1 pseudo-testcross (3010 × CW 1010) population. These QTLs were detected only for a single location and a single year. The symbols used in this table have exactly same abbreviations as given for table 2.5 and 2.6.

Trait	Parent	QTL code	Chr.	Year/ Location	Peak Marker	Peak LOD	R^2	Allele dir.	LSI (cM)	Flanking Markers
FD	3010	dorm8	1A	β1	TP73186	7.7	0.12	(-)	69.1-71.3	TP73186 – TP70400
FD	3010	dorm9	5A	π1	MRG_28485316	5.3	0.08	(-)	36.1-45.8	MRG_28485316 - TP63204
FD	3010	dorm10	6D	π3	MRG_2402742	4.7	0.08	(-)	16.7-24.0	MRG_2402742 - TP18699
FD	3010	dorm11	4D	π2	TP64707	4.5	0.08	(-)	101.3-101.9	TP64707 - TP61536
FD	3010	dorm12	1C	π2	TP78612	3.4	0.08	(-)	62-70.3	TP78612 - TP68882
FD	3010	dorm13	7B	β4	TP43449	4.2	0.07	(-)	15.4-18.5	TP14416 - TP25746
FD	3010	dorm14	2C	π2	TP29084	3.8	0.07	(-)	32.7-39.5	TP29084 -TP82709
FD	3010	dorm15	4C	π4	TP64526	4.2	0.06	(-)	16.3-18.8	MRG_18042076 - TP64526
FD	3010	dorm16	2B	π3	TP69826	4.0	0.06	(-)	25.3-28.7	TP78664 - TP59834
FD	3010	dorm17	5A	β1	TP63107	3.8	0.06	(-)	24.1 – 27.6	TP89078 – TP46688
FD	3010	dorm18	3A	β1	TP32175	3.3	0.05	(-)	9-15	TP32175 – TP44970
FD	3010	dorm19	3D	π3	TP67190	3.2	0.05	(-)	5.6-13.3	TP67190 - TP58690
FD	3010	dorm20	3A	β2	TP32136	3.1	0.05	(-)	18.6-22	TP48316 - MRG_4754683

FD	3010	dorm21	7D	β4	TP32437	3.1	0.05	(-)	5.2-7.7	TP79530 - TP53493
FD	3010	dorm22	7C	π2	MRG_30285700	3.1	0.05	(-)	19.9-22.4	MRG_30285700 - TP49176
FD	3010	dorm23	5D	π1	TP31552	3.2	0.04	(-)	36.5-41.6	TP31552 - TP28126
FD	CW 1010	ndorm12	4D	β4	TP32802	6.7	0.12	(+)	64.5-66.1	TP32802 - TP5506
FD	CW 1010	ndorm13	7A	β2	TP63954	5.9	0.10	(-)	28.1-29.4	TP63954 - TP87998
FD	CW 1010	ndorm14	4B	β4	TP11836	3.8	0.05	(+)	71.2-72.4	TP11836 - TP10328
FD	CW 1010	ndorm15	7A	β1	MRG_31595966	3.2	0.04	(+)	60.1 – 61.4	MRG_7180813 - TP51152
WH	3010	wh9	7C	ψ2	TP84244	4.9	0.09	(-)	72.7 - 73.9	TP84244 - TP44147
WH	3010	wh10	7C	λ1	TP74326	4.5	0.09	(+)	106.5 - 110.6	TP74326 - TP30485
WH	3010	wh11	8B	ψ3	TP34659	3.9	0.08	(+)	43.3 - 47	TP24160 - TP34659
WH	3010	wh12	8D	ψ3	TP15842	3.7	0.07	(+)	77 - 80.1	TP33611 - TP15842
WH	3010	wh13	3B	λ1	TP63723	3.2	0.06	(+)	44.2 - 50.3	TP63723 - TP46610
WH	3010	wh14	3D	ψ2	TP26775	3.3	0.06	(+)	28.5 - 33.6	TP88373 - TP16429
WH	3010	wh15	2B	λ1	TP6025	3.1	0.05	(+)	8.7-11.1	TP19047 - TP6025
WH	3010	wh16	2C	λ3	TP29084	3.2	0.05	(+)	33.5 - 40	TP29084 - TP82709
WH	CW 1010	ws10	4D	λ1	TP88199	10.1	0.18	(-)	33.9 - 37.8	TP54779 - TP88199
WH	CW 1010	ws11	1B	ψ2	TP66690	3.8	0.076	(+)	41.8 - 43.1	TP64641 - TP66690
WH	CW 1010	ws12	5A	ψ3	TP33164	3.9	0.06	(-)	62.7 - 63.6	TP33164 - TP30048
WH	CW 1010	ws13	1B	ψ2	TP7086	3.1	0.07	(-)	22.9 - 24.1	TP7086 - TP65701

WH	CW 1010	ws14	8A	ψ3	MRG_12811807	3.3	0.05	(-)	20.1 - 20.3	MRG_12811807 - TP29734
WH	CW 1010	ws15	1A	λ3	TP81842	3.7	0.05	(+)	54.6 - 55.8	TP6332 - TP81842
WH	CW 1010	ws16	6A	λ3	TP60069	3.4	0.04	(-)	52.5 - 54.7	TP60069 - TP5275

Table 2.8. Potential QTLs for dormancy and WH identified in a pseudo-testcross F1 population (3010 × CW 1010) using phenotypic data generated across two environments (JPC and BVL).

Trait	Parent	QTL code	Chr.	Year	Peak Marker	Peak LOD	R^2	Allele dir.	LSI (cM)	Flanking Markers
FD	3010	dorm24	3A	$4	TP67563	5.4	0.09	(-)	49.4 – 51.8	TP71671 - TP76041
FD	3010	dorm25	3D	$4	MRG_38650252	3.6	0.09	(-)	70.1 – 74.4	MRG_31477229 - TP57603
FD	3010	dorm26	3D	$2	TP16817	4.3	0.09	(-)	76.3 – 79.5	TP16817 - TP5092
FD	3010	dorm27	1C	$1	TP32721	4.8	0.07	(-)	81.8 – 85.1	TP32721 - TP40620
FD	3010	dorm28	1A	$3	TP46942	3.6	0.06	(-)	72 - 73	TP72089 - TP73780
FD	CW 1010	ndorm16	4D	$3	TP69818	3.1	0.09	(+)	8.3- 10.7	TP69818 - TP82286
FD	CW 1010	ndorm17	4D	$2	TP80681	3.9	0.08	(+)	19.7 – 23.5	TP80681 - TP83595
WH	3010	wh17	4C	ϕ1	TP81375	4.6	0.10	(+)	37.6 – 39.5	TP80478 - TP81375
WH	3010	wh18	3D	ϕ3	TP43991	5.1	0.09	(+)	58.9-63.2	TP50808 - TP87713
WH	3010	wh19	7A	ϕ3	TP71458	3.7	0.06	(+)	50.5 - 52	TP15177 - TP71458

Across environment dormancy: $1 = 2015, fall; $2 = 2016, fall; $3 = 2016, winter; $4 = 2017, winter;

Across environment winter hardiness: ϕ1 = 2015; ϕ2 = 2016; ϕ3 = 2017

Other abbreviations: Chr. = Chromosome; Dir. = Direction; LSI = 1-LOD support interval in cM

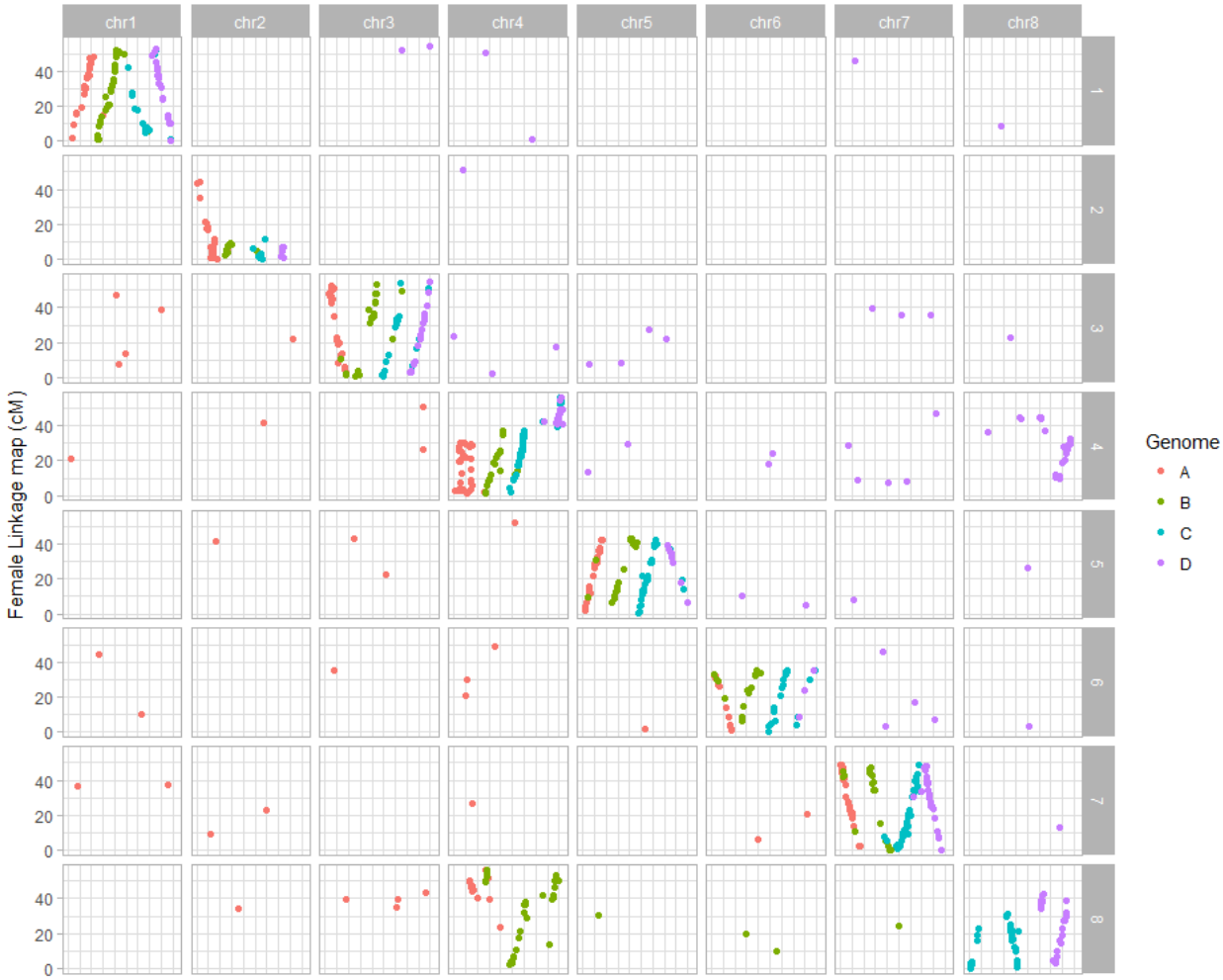


Figure 2.1. Dot plot displaying the grouping pattern and positions of SNPs on 32 linkage groups of alfalfa 3010 linkage map. Of the 32 groups, each four homologs groups were assigned to a chromosome based on synteny with the *Medicago truncatula* genome.

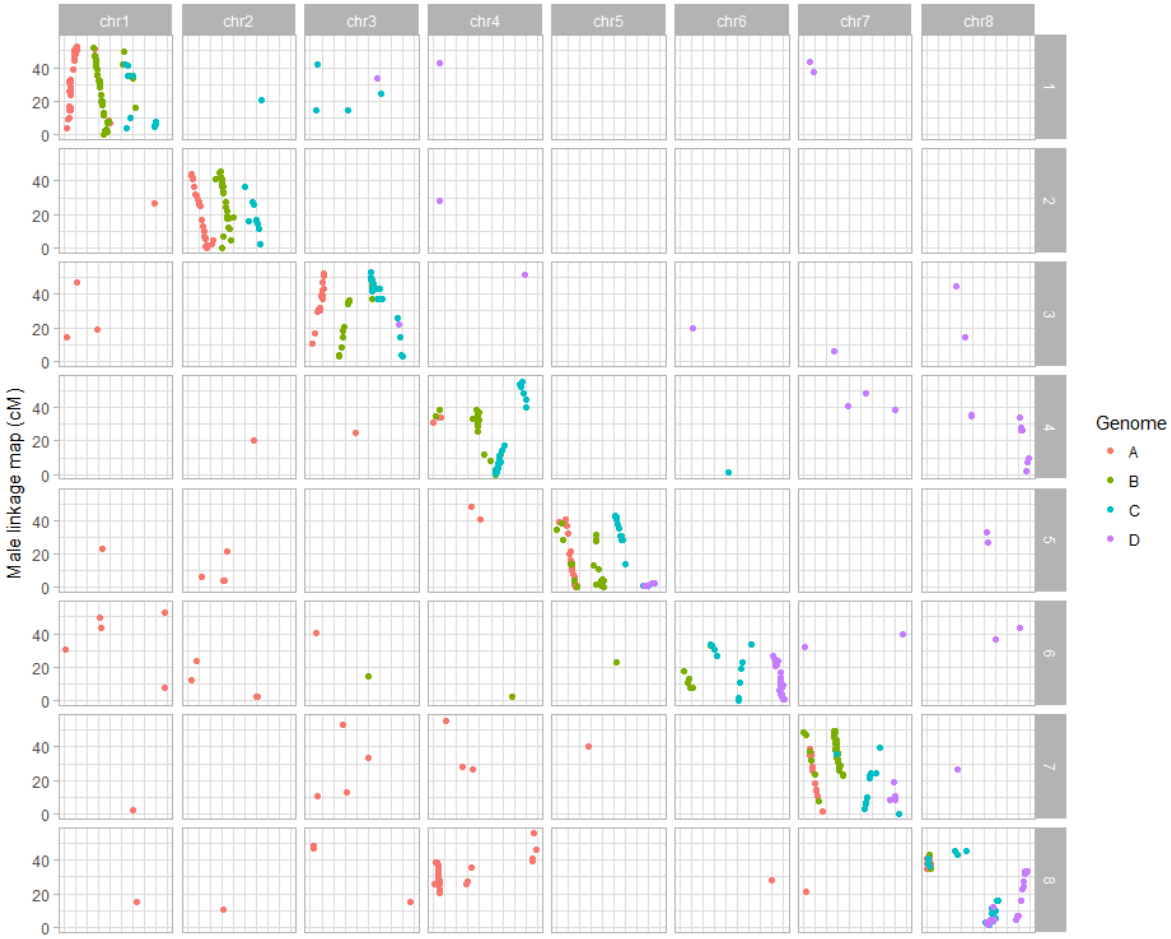


Figure 2.2. Dot plot displaying the grouping pattern and positions of SNPs on 32 linkage groups of alfalfa CW 1010 linkage map. Of the 32 groups, each four homologs groups were assigned to a chromosome based on synteny with the *Medicago truncatula* genome.

Figure 2.3. Image showing dormant (left) and non-dormant (right) progeny rows from the pseudo-testcross F1 population (3010 × CW 1010) after frost occurrence in early March 2017 at the JPC environment. Frost damage symptoms are clearly visible on the non-dormant progeny.

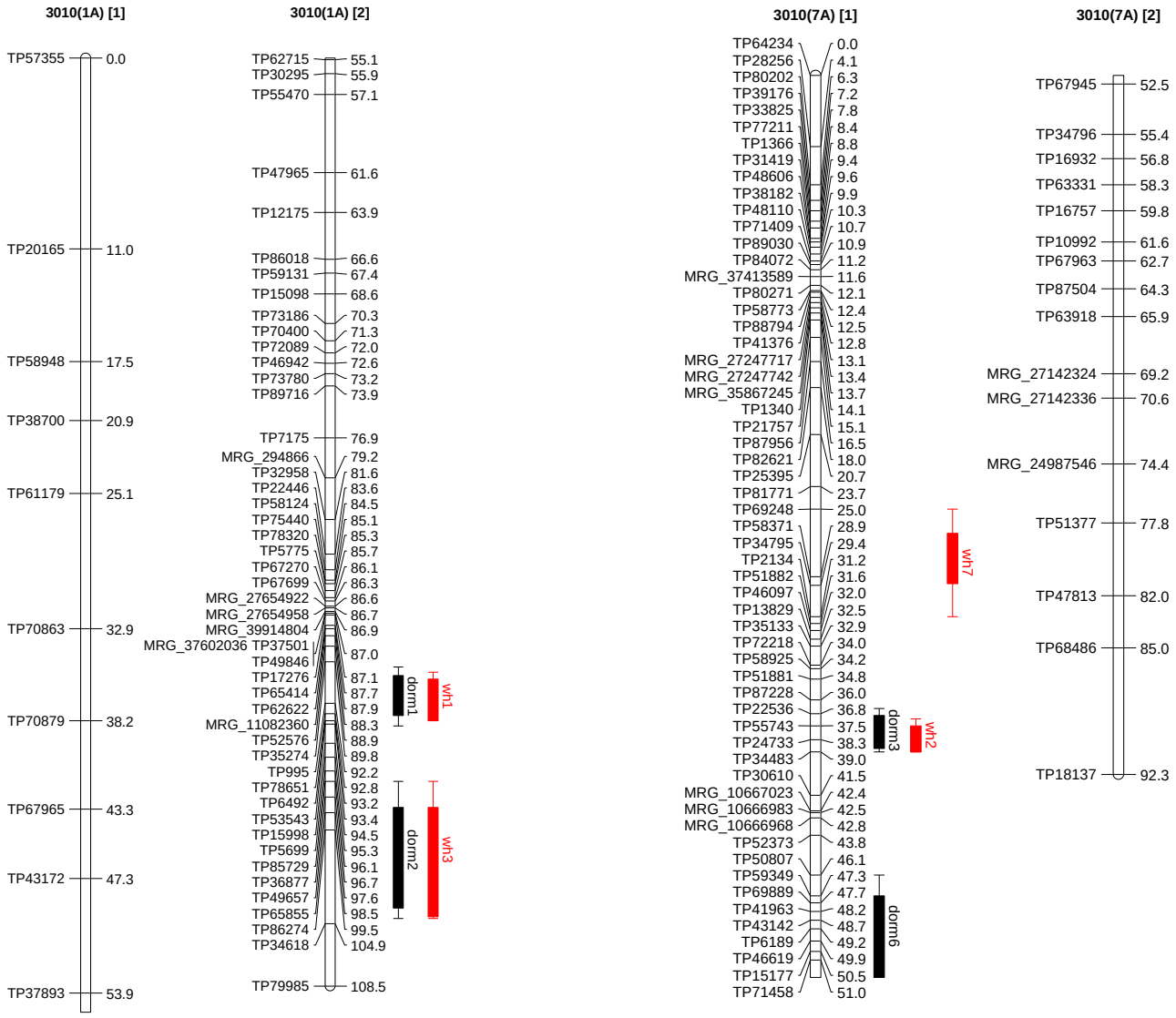


Figure 2.4. Dormancy (black bar) and WH (red bar) stable QTLs mapped on linkage maps of homolog 1A (left) and 7A (right) for 3010 parent. The QTL bars have two intervals, an inner (1-LOD support) interval and an outer (2-LOD support) interval, where the rectangle represents inner interval and the line represents the outer. Some stable QTLs for dormancy were co-localized with WH in the same genomic regions.

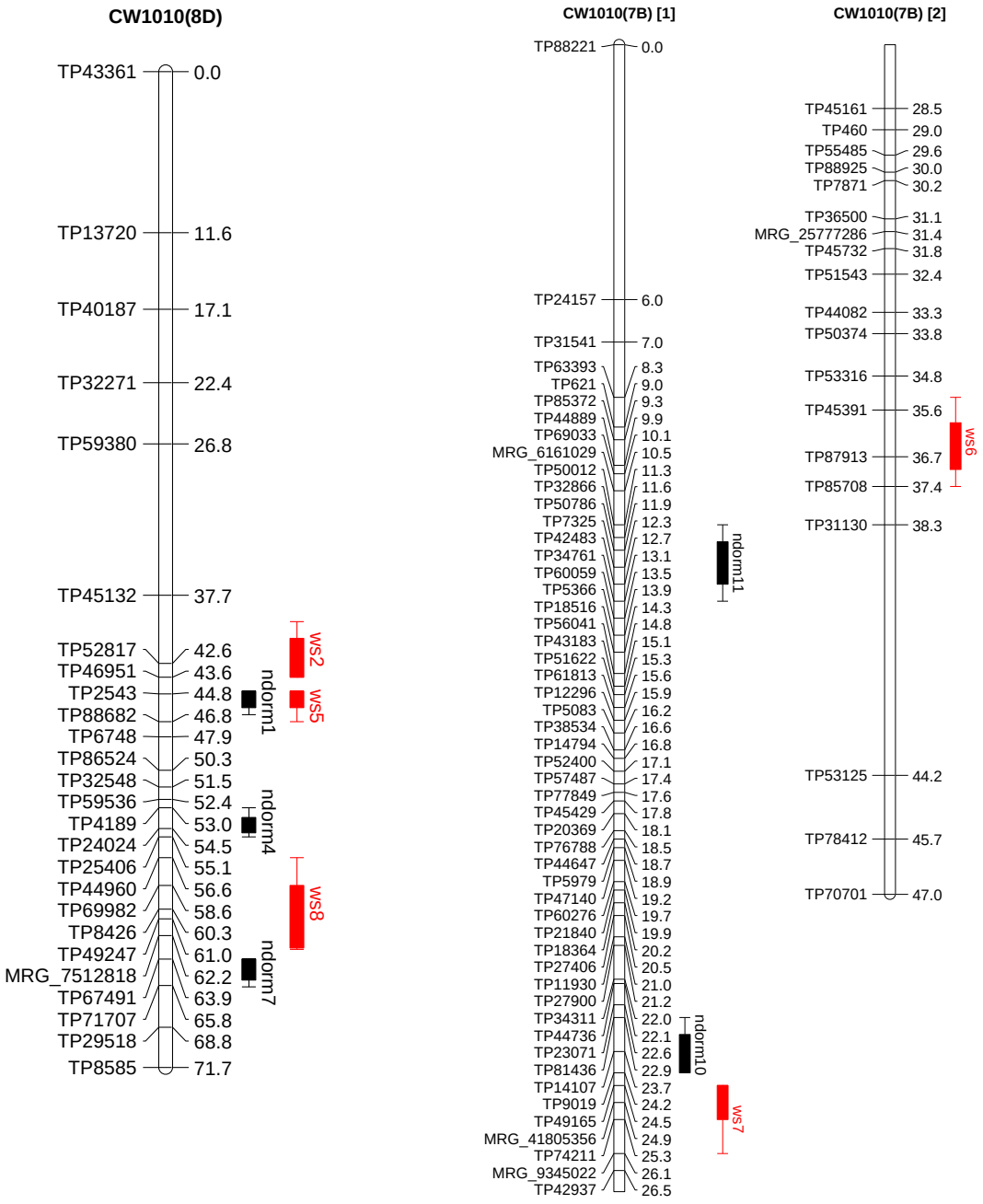


Figure 2.5. Dormancy (black bar) and WH (red bar) stable QTLs mapped on linkage maps of homolog 8D (left) and 7B (right) for CW 1010 parent. The QTL bars have two intervals, an inner (1-LOD support) interval and an outer (2-LOD support) interval, where the rectangle represents inner interval and the line represents the outer. Some stable QTLs for dormancy were co-localized with WH in the same genomic regions.

References

1. **Adhikari L, Missaoui AM** (2017) Nodulation Response to Molybdenum Supplementation in Alfalfa and it's Correlation with Root and Shoot Growh in Low Ph Soil. Journal of Plant Nutrition 40:2290-2302. doi:10.1080/01904167.2016.1264601

2. **Duke J** (1981) Handbook of legumes of world economic importance. New York , NY:Plenum Press

3. **Li X, Brummer EC** (2012) Applied Genetics and Genomics in Alfalfa Breeding. Agronomy 2 (1):40

4. http://nmsp.cals.cornell.edu/publications/factsheets/factsheet39.pdf Cornell University. 2008

5. http://usda.mannlib.cornell.edu/usda/current/CropProdSu/CropProdSu-01-12-2018.pdf

6. **Adhikari L, Razar RM, Paudel D, Ding R, Missaoui AM** (2017) Insights into Seasonal Dormancy of Perennial Herbaceous Forages. American Journal of Plant Sciences 8 (11):2650

7. **Teuber L, Taggard K, Gibbs L, McCaslin M, Peterson M, Barnes D** (1998) Fall dormancy. Standard tests to characterize alfalfa cultivars. North American Alfalfa Improve. Conf., 36th, Bozeman, MT, ate 1998. pp 2-6

8. https://www.alfalfa.org/pdf/2017%20NAFA%20Variety%20Leaflet.pdf

9. **McKenzie JS, Paquin R, Duke SH** (1988) Cold and Heat Tolerance. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and Alfalfa Improvement. Agronomy Monograph, vol 29. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, pp 259-302. doi:10.2134/agronmonogr29.c8

10. **Brummer EC, Shah MM, Luth D** (2000) Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. Crop Science 40 (4):971-977

11. **Xiong Y, Fei S-z, Arora R, Brummer EC, Barker RE, Jung G, Warnke SE** (2007) Identification of quantitative trait loci controlling winter hardiness in an annual × perennial ryegrass interspecific hybrid population. Molecular Breeding 19 (2):125-136. doi:10.1007/s11032-006-9050-1

12. **Olsen JE** (2010) Light and temperature sensing and signaling in induction of bud dormancy in woody plants. Plant Molecular Biology 73 (1):37-47. doi:10.1007/s11103-010-9620-9

13. **Tanino KK, Kalcsits L, Silim S, Kendall E, Gray GR** (2010) Temperature-driven plasticity in growth cessation and dormancy development in deciduous woody plants: a working hypothesis suggesting how molecular and cellular function is affected by temperature during dormancy induction. Plant Molecular Biology 73 (1):49-65. doi:10.1007/s11103-010-9610-y

14. **Ibáñez C, Kozarewa I, Johansson M, Ögren E, Rohde A, Eriksson ME** (2010) Circadian Clock Components Regulate Entry and Affect Exit of Seasonal Dormancy as Well as Winter Hardiness in Populus Trees. Plant Physiology 153 (4):1823-1833. doi:10.1104/pp.110.158220

15. **Brouwer DJ, Duke SH, Osborn TC** (2000) Mapping genetic factors associated with winter hardiness, fall growth, and freezing injury in autotetraploid alfalfa. Crop Science 40 (5):1387-1396

16. **Li X, Han Y, Wei Y, Acharya A, Farmer AD, Ho J, Monteros MJ, Brummer EC** (2014a) Development of an Alfalfa SNP Array and Its Use to Evaluate Patterns of Population Structure and Linkage Disequilibrium. PLOS ONE 9 (1):e84329. doi:10.1371/journal.pone.0084329

17. **STOUT DG, HALL JW** (1989) Fall growth and winter survival of alfalfa in interior British Columbia. Canadian Journal of Plant Science 69 (2):491-499

18. **Li X, Alarcón-Zúñiga B, Kang J, Nadeem Tahir MH, Jiang Q, Wei Y, Reyno R, Robins JG, Brummer EC** (2015) Mapping Fall Dormancy and Winter Injury in Tetraploid Alfalfa. Crop Science 55 (5):1995-2011. doi:10.2135/cropsci2014.12.0834

19. **Cunningham S, Volenec J, Teuber L** (1998) Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. Crop Science 38 (4):962-969

20. **Shu Y, Li W, Zhao J, Zhang S, Xu H, Liu Y, Guo C** (2017) Transcriptome sequencing analysis of alfalfa reveals CBF genes potentially playing important roles in response to freezing stress. Genetics and molecular biology (AHEAD):0-0

21. **Zhang S, Shi Y, Cheng N, Du H, Fan W, Wang C** (2015) De Novo Characterization of Fall Dormant and Nondormant Alfalfa (Medicago sativa L.) Leaf Transcriptome and Identification of Candidate Genes Related to Fall Dormancy. PloS one 10 (3)

22. **Castonguay Y, Bertrand A, Michaud R, Laberge S** (2011) Cold-induced biochemical and molecular changes in alfalfa populations selectively improved for freezing tolerance. Crop Science 51 (5):2132-2144

23. **Dhanaraj AL, Alkharouf NW, Beard HS, Chouikha IB, Matthews BF, Wei H, Arora R, Rowland LJ** (2007) Major differences observed in transcript profiles of blueberry during cold acclimation under field and cold room conditions. Planta 225 (3):735-751. doi:10.1007/s00425-006-0382-1

24. **Gusta LV, Wisniewski M** (2013) Understanding plant cold hardiness: an opinion. Physiol Plant 147 (1):4-14. doi:10.1111/j.1399-3054.2012.01611.x

25. **Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE** (2011) A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. PLoS ONE 6 (5):e19379. doi:10.1371/journal.pone.0019379

26. **Boudhrioua C, Bastien M, Légaré G, Pomerleau S, St-Cyr J, Boyle B, Belzile F** (2017) Genotyping-by-Sequencing in Potato. In: Kumar Chakrabarti S, Xie C, Kumar Tiwari J (eds) The Potato Genome. Springer International Publishing, Cham, pp 283-296. doi:10.1007/978-3-319-66135-3_15

27. **Gar O, Sargent DJ, Tsai C-J, Pleban T, Shalev G, Byrne DH, Zamir D** (2011) An Autotetraploid Linkage Map of Rose (Rosa hybrida) Validated Using the Strawberry (Fragaria vesca) Genome Sequence. PLOS ONE 6 (5):e20463. doi:10.1371/journal.pone.0020463

28. **Li X, Wei Y, Acharya A, Jiang Q, Kang J, Brummer EC** (2014b) A saturated genetic linkage map of autotetraploid alfalfa (Medicago sativa L.) developed using genotyping-by-sequencing is highly syntenous with the Medicago truncatula genome. G3 (Bethesda) 4 (10):1971-1979

29. **Hackett CA, Milne I, Bradshaw JE, Luo Z** (2007) TetraploidMap for Windows: linkage map construction and QTL mapping in autotetraploid species. The Journal of heredity 98 (7):727-729. doi:10.1093/jhered/esm086

30. **Hackett CA, Boskamp B, Vogogias A, Preedy KF, Milne I** (2017) TetraploidSNPMap: Software for Linkage Analysis and QTL Mapping in Autotetraploid Populations Using SNP Dosage Data. Journal of Heredity 108 (4):438-442. doi:10.1093/jhered/esx022

31. **da Silva WL, Ingram J, Hackett CA, Coombs JJ, Douches D, Bryan GJ, De Jong W, Gray S** (2017) Mapping Loci That Control Tuber and Foliar Symptoms Caused by PVY in Autotetraploid Potato (Solanum tuberosum L.). G3: Genes, Genomes, Genetics 7 (11):3587-3595

32. https://www.kyazma.nl/docs/JM5Features.pdf

33. **Eujayl I, Sledge MK, Wang L, May GD, Chekhovskiy K, Zwonitzer JC, Mian MAR** (2004) Medicago truncatula EST-SSRs reveal cross-species genetic markers for Medicago spp. Theoretical & Applied Genetics 108 (3):414-422. doi:10.1007/s00122-003-1450-6

34. **Li X, Wei Y, Moore KJ, Michaud R, Viands DR, Hansen JL, Acharya A, Brummer EC** (2011) Association Mapping of Biomass Yield and Stem Composition in a Tetraploid Alfalfa Breeding Population. The Plant Genome 4 (1):24-35. doi:10.3835/plantgenome2010.09.0022

35. https://www.alfalfa.org/pdf/2014%20NAFA%20Variety%20Leaflet.pdf

36. **Doyle J, Doyle J** (1987) Genomic plant DNA preparation from fresh tissue-CTAB method. Phytochem Bull 19 (11):11-15

37. **Melo ATO, Bartaula R, Hale I** (2016) GBS-SNP-CROP: a reference-optional pipeline for SNP discovery and plant germplasm characterization using variable length, paired-end genotyping-by-sequencing data. BMC Bioinformatics 17 (1):29. doi:10.1186/s12859-016-0879-y

38. **Lu F, Lipka AE, Glaubitz J, Elshire R, Cherney JH, Casler MD, Buckler ES, Costich DE** (2013) Switchgrass genomic diversity, ploidy, and evolution: novel insights from a network-based SNP discovery protocol. PLoS genetics 9 (1):e1003215

39. **Bolger A, Giorgi F** (2014) Trimmomatic: a flexible read trimming tool for illumina NGS data. URL http://www usadellab org/cms/index php

40. http://bio-bwa.sourceforge.net/

41. **Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R** (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics 25 (16):2078-2079. doi:10.1093/bioinformatics/btp352

42. SAS Institute Inc. (2004). Cary, NC: SAS Institute Inc

Institute Inc

43. **McCaslin M, Woodward T, Undersander D** (2013) Winter survival. Standard tests to characterize alfalfa cultivars. North America Alfalfa Improvement Conference, Beltsville, MD. Available at http://www. naaic. org/stdtests/wintersurvivalnew. htm (verified 20 May 2013), ate 2003.

44. **Haggard JE, Johnson EB, St. Clair DA** (2015) Multiple QTL for Horticultural Traits and Quantitative Resistance to Phytophthora infestans Linked on Solanum habrochaites Chromosome 11. G3: Genes|Genomes|Genetics 5 (2):219-233. doi:10.1534/g3.114.014654

45. **Wan S-M, Liu H, Zhao B-W, Nie C-H, Wang W-M, Gao Z-X** (2017) Construction of a high-density linkage map and fine mapping of QTLs for growth and gonad related traits in blunt snout bream. Scientific Reports 7:46509.doi:10.1038/srep46509https://www.nature.com/articles/srep46509#supplementary-information

46. **Voorrips RE** (2002) MapChart: Software for the graphical presentation of linkage maps and QTLs. The Journal of Heredity 93 (1): 77-78

47. **Adhikari L, Mohseni-Moghadam M, Missaoui A** (2018) Allelopathic Effects of Cereal Rye on Weed Suppression and Forage Yield in Alfalfa. American Journal of Plant Sciences Vol.09No.04:685-700. doi:10.4236/ajps.2018.94054

48. **Moriguchi Y, Ujino-Ihara T, Uchiyama K, Futamura N, Saito M, Ueno S, Matsumoto A, Tani N, Taira H, Shinohara K, Tsumura Y** (2012) The construction of a high-density linkage map for identifying SNP markers that are tightly linked to a nuclear-recessive major gene for male sterility in Cryptomeria japonica D. Don. BMC Genomics 13 (1):95. doi:10.1186/1471-2164-13-95

49. **Julier B, Flajoulot S, Barre P, Cardinet G, Santoni S, Huguet T, Huyghe C** (2003) Construction of two genetic linkage maps in cultivated tetraploid alfalfa (Medicago sativa) using microsatellite and AFLP markers. BMC Plant Biology 3:9-9. doi:10.1186/1471-2229-3-9

50. **Brouwer DJ, Osborn TC** (1999) A molecular marker linkage map of tetraploid alfalfa (Medicago sativa L.). Theoretical and Applied Genetics 99 (7):1194-1200. doi:10.1007/s001220051324

51. **Musial JM, Mackie JM, Armour DJ, Phan HT, Ellwood SE, Aitken KS, Irwin JA** (2007) Identification of QTL for resistance and susceptibility to Stagonospora meliloti in autotetraploid lucerne. TAG Theoretical and applied genetics Theoretische und angewandte Genetik 114 (8):1427-1435. doi:10.1007/s00122-007-0528-y

52. **Kearsey MJ, Ramsay LD, Jennings DE, Lydiate DJ, Bohuon EJR, Marshall DF** (1996) Higher recombination frequencies in female compared to male meisoses in Brassica oleracea. Theoretical and Applied Genetics 92 (3):363-367. doi:10.1007/bf00223680

53. **Gorjanc G, Cleveland MA, Houston RD, Hickey JM** (2015) Potential of genotyping-by-sequencing for genomic selection in livestock populations. Genetics, Selection, Evolution : GSE 47 (1):12. doi:10.1186/s12711-015-0102-z

54. **Wu K, Burnquist W, Sorrells M, Tew T, Moore P, Tanksley S** (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theoretical and Applied Genetics 83 (3):294-300

55. **Scott LJ** (2004) Implications of evolutionary history and population structure for the analysis of quantitative trait loci in the ancient conifer Araucaria cunninghamii. Southern Cross University, Lismore, NSW (PhD thesis)

56. **Wu S, Yang J, Huang Y, Li Y, Yin T, Wullschleger SD, Tuskan GA, Wu R** (2010) An improved approach for mapping quantitative trait Loci in a pseudo-testcross: revisiting a poplar mapping study. Bioinformatics and biology insights 4:1-8

57. **Grattapaglia D, Sederoff R** (1994) Genetic linkage maps of Eucalyptus grandis and Eucalyptus urophylla using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137 (4):1121-1137

58. **Shepherd M, Cross M, Dieters MJ, Henry R** (2003) Genetic maps for Pinus elliottii var. elliottii and P. caribaea var. hondurensis using AFLP and microsatellite markers. Theoretical and Applied Genetics 106 (8):1409-1419. doi:10.1007/s00122-002-1185-9

59. **Xie W, Zhang X, Cai H, Huang L, Peng Y, Ma X** (2010) Genetic maps of SSR and SRAP markers in diploid orchardgrass (Dactylis glomerata L.) using the pseudo-testcross strategy. Genome 54 (3):212-221. doi:10.1139/G10-111

60. **Pastina M, Malosetti M, Gazaffi R, Mollinari M, Margarido G, Oliveira K, Pinto L, Souza A, Van Eeuwijk F, Garcia A** (2012) A mixed model QTL analysis for sugarcane multiple-harvest-location trial data. Theoretical and Applied Genetics 124 (5):835-849

61. **Weller JI** (1992) Statistical methodologies for mapping and analysis of quantitative trait loci. Plant Genomes: Methods for Genetic and Physical Mapping. Springer, pp 181-207

62. **Boopathi NM** (2013) Genetic mapping and marker assisted selection: basics, practice and benefits. New Delhi: Springer India

63. **Semagn K, Bjørnstad Å, Xu Y** (2010) The genetic dissection of quantitative traits in crops. Electronic Journal of Biotechnology 13 (5):16-17

64. **Zúñiga BA, Scott P, Moore K, Luth D, Brummer E** (2004) Quantitative trait locus mapping of winter hardiness metabolites in autotetraploid alfalfa (M. sativa). Molecular breeding of forage and turf. Springer, pp 97-104

65. **Robins J, Luth D, Campbell T, Bauchan G, He C, Viands D, Hansen J, Brummer E** (2007) Genetic mapping of biomass production in tetraploid alfalfa. Crop Sci 47:1 - 10

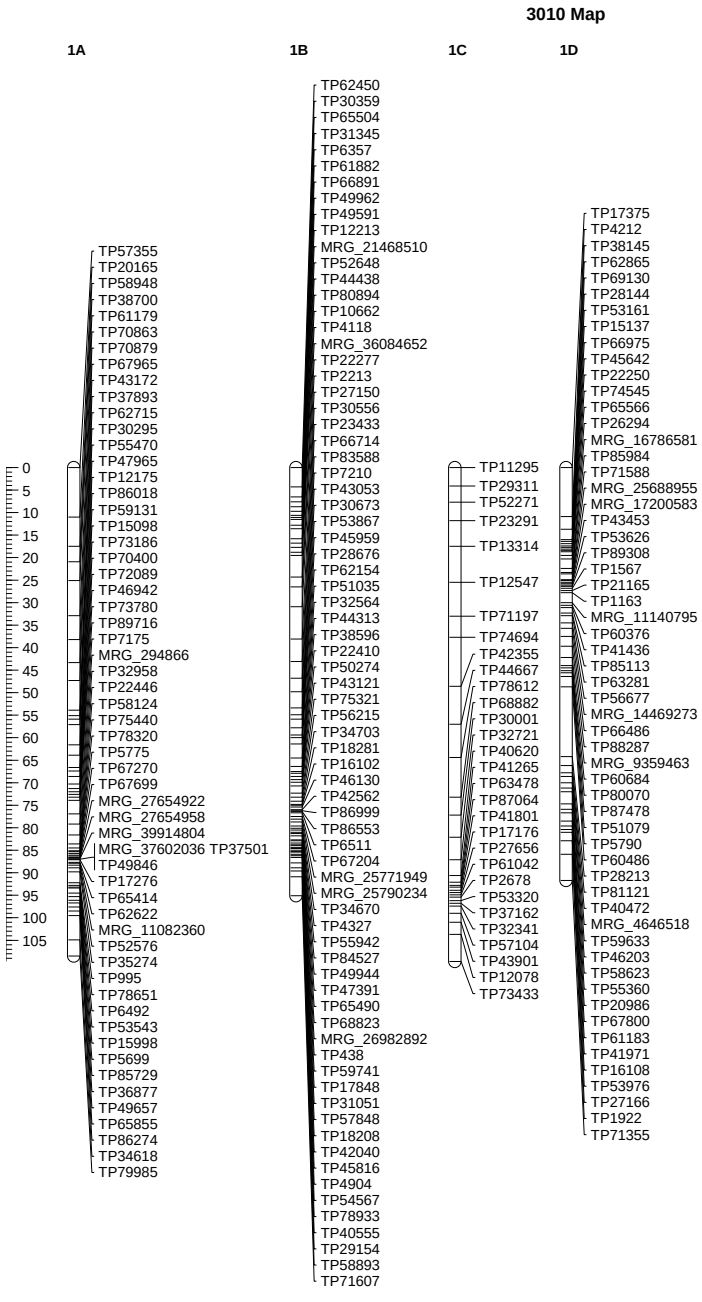
66. **El-Soda M, Malosetti M, Zwaan BJ, Koornneef M, Aarts MGM** Genotype × environment interaction QTL mapping in plants: lessons from Arabidopsis. Trends in Plant Science 19 (6):390-398. doi:10.1016/j.tplants.2014.01.001

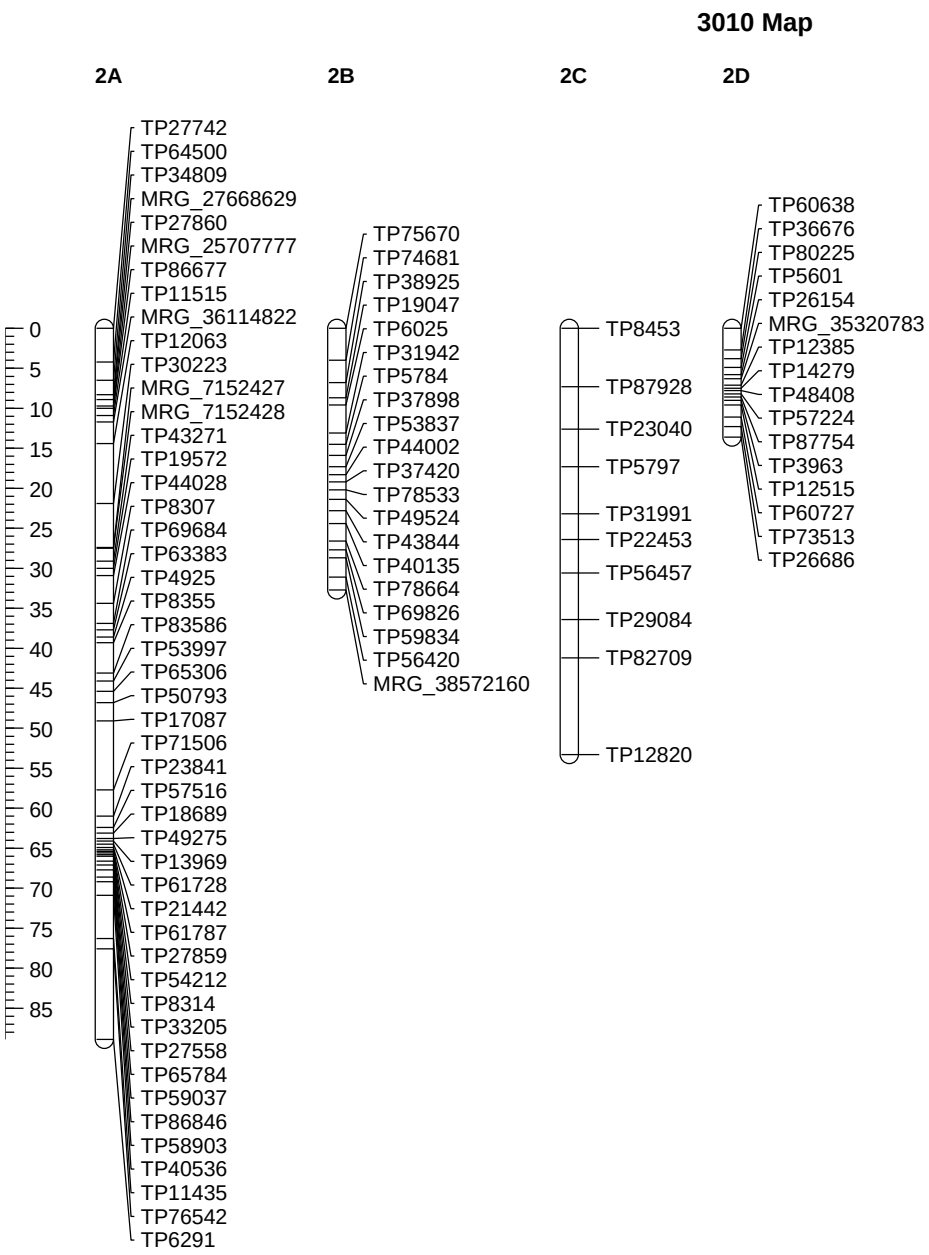
67. **Perry MC, McIntosh MS, Wiebold WJ, Welterlen M** (1987) Genetic analysis of cold hardiness and dormancy in alfalfa. Genome 29 (1):144-149. doi:10.1139/g87-024

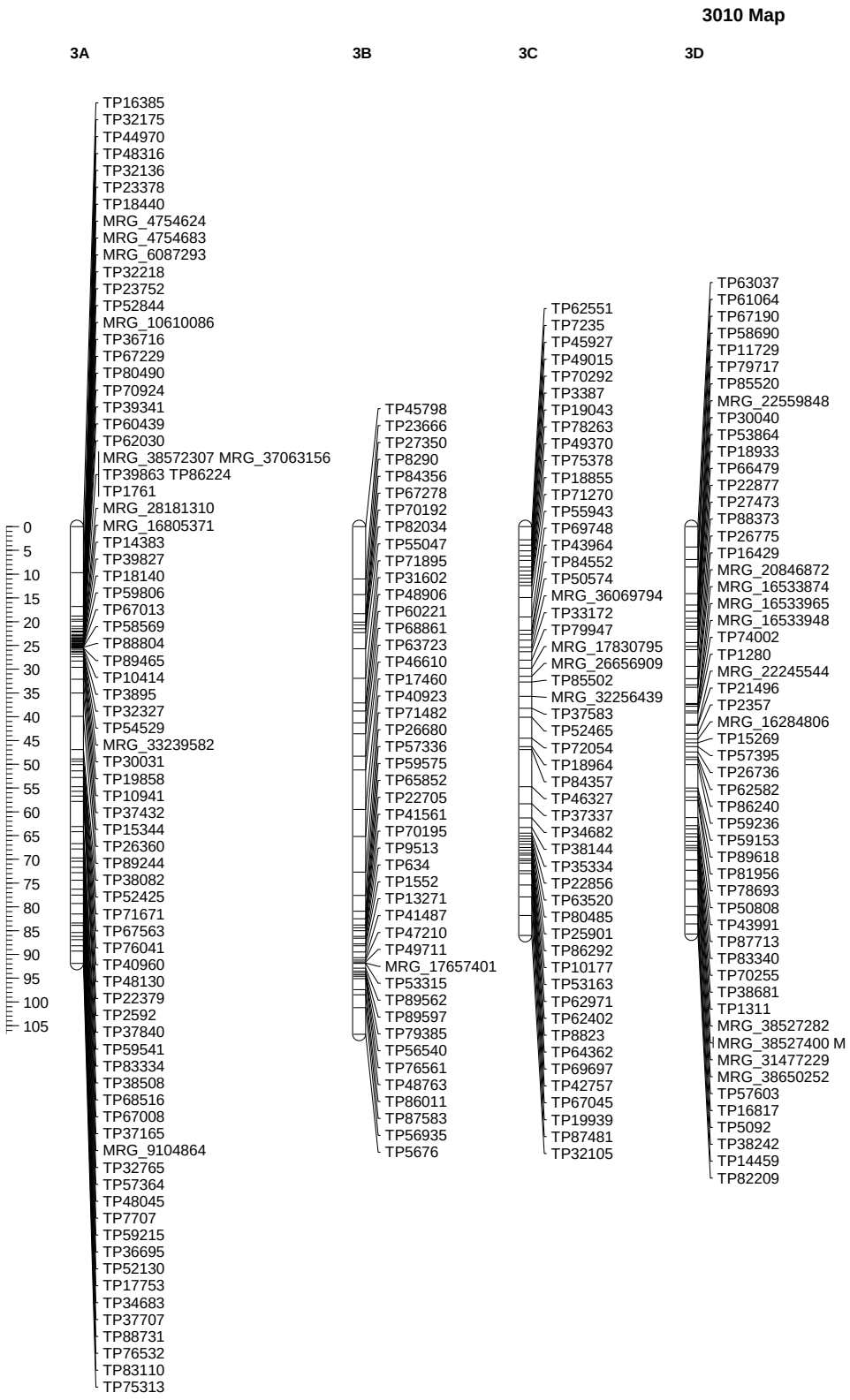
68. **Weishaar MA, Brummer EC, Volenec JJ, Moore KJ, Cunningham S** (2005) Improving Winter Hardiness in Nondormant Alfalfa Germplasm This journal paper of the Iowa Agric. Home Econ. Exp. Stn., Ames, IA, Project No. 6631 and 6525, was supported by the Hatch Act and State of Iowa funds. Crop Science 45 (1):60-65. doi:10.2135/cropsci2005.0060

Appendix

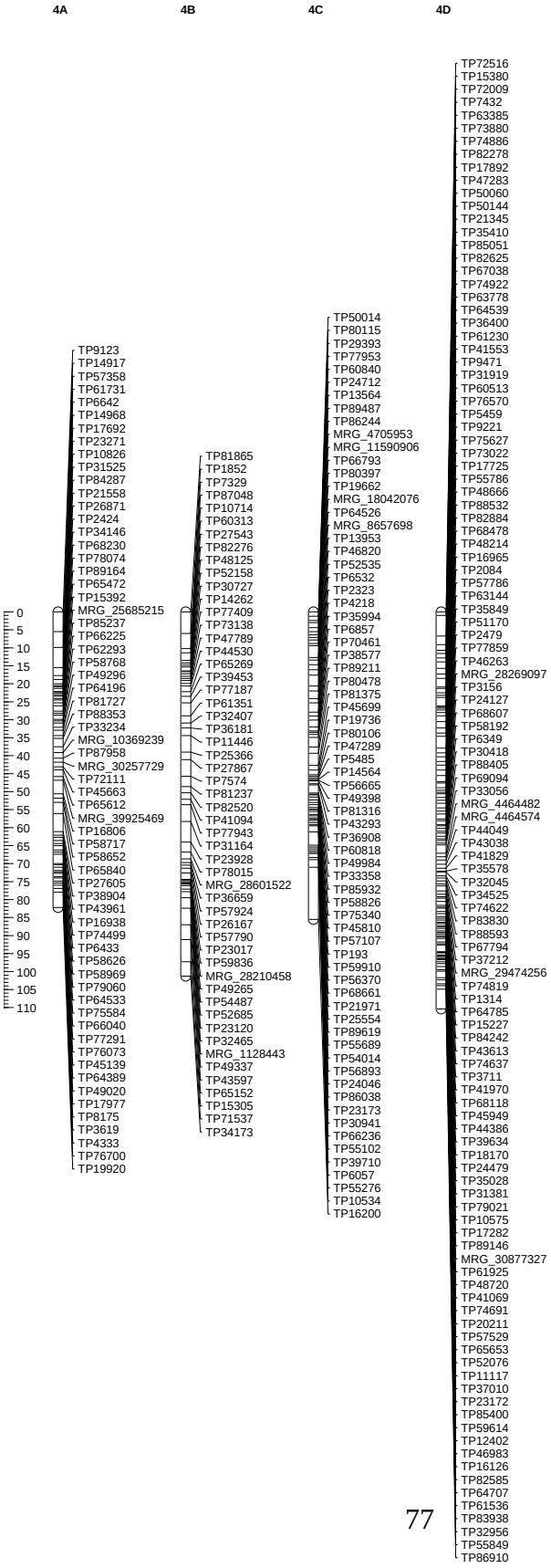
Supplemental figures

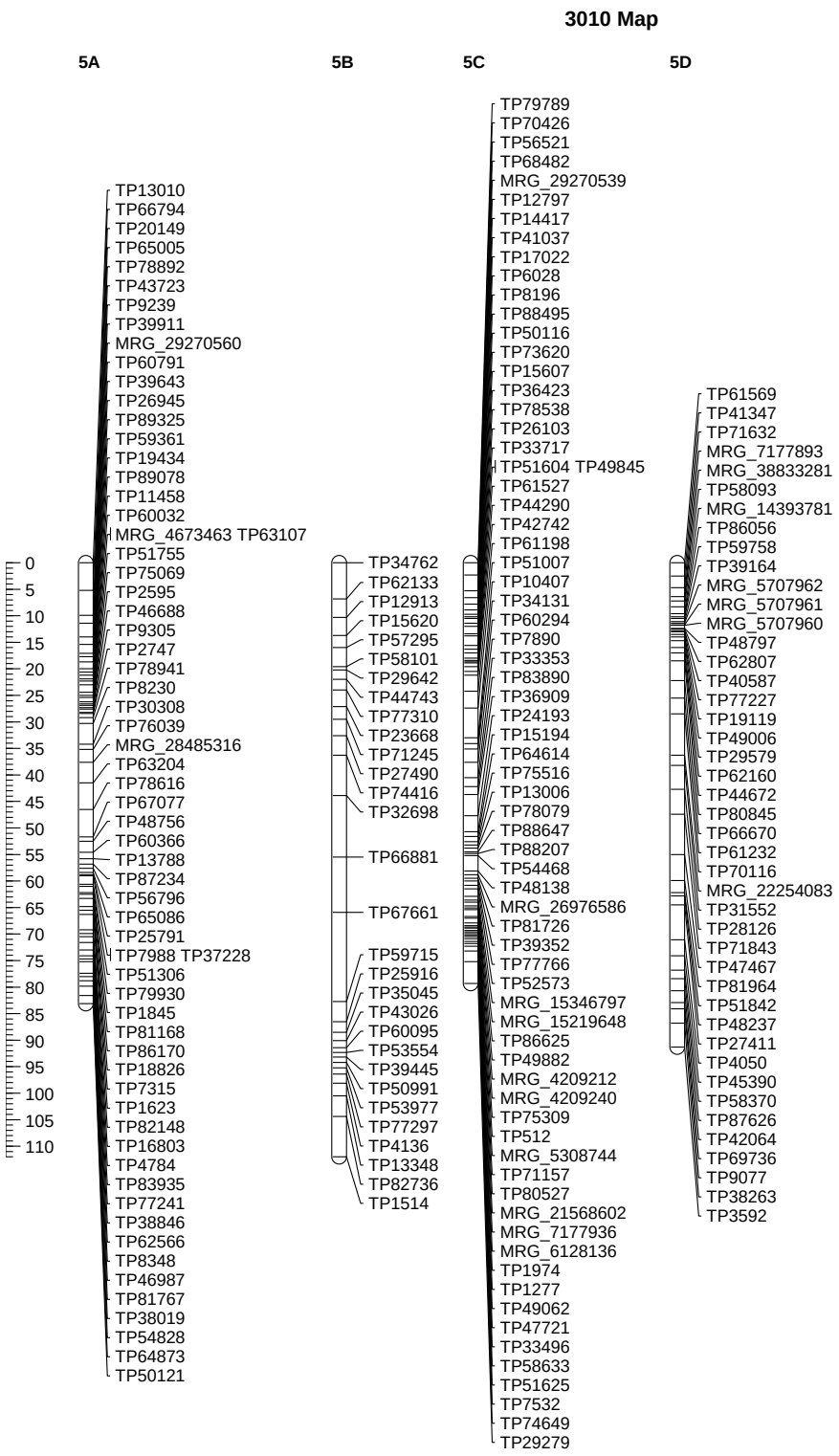


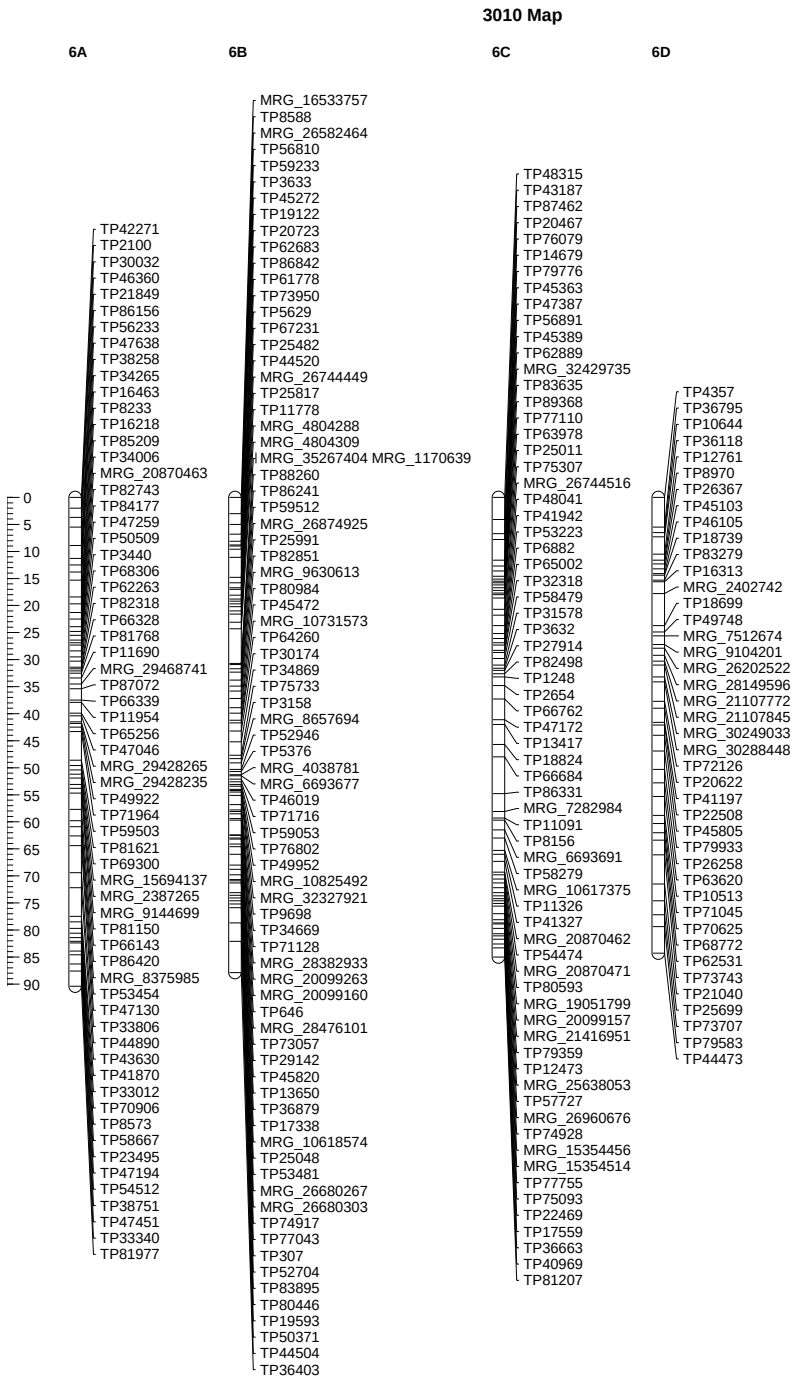

3010 Map
2A
2B
2C
2D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
TP27742
TP64500
TP34809
MRG_27668629
TP27860
MRG_25707777
TP86677
TP11515
MRG_36114822
TP12063
TP30223
MRG_7152427
MRG_7152428
TP43271
TP19572
TP44028
TP8307
TP69684
TP63383
TP4925
TP8355
TP83586
TP53997
TP65306
TP50793
TP17087
TP71506
TP23841
TP57516
TP18689
TP49275
TP13969
TP61728
TP21442
TP61787
TP27859
TP54212
TP8314
TP33205
TP27558
TP65784
TP59037
TP86846
TP58903
TP40536
TP11435
TP76542
TP6291
TP75670
TP74681
TP38925
TP19047
TP6025
TP31942
TP5784
TP37898
TP53837
TP44002
TP37420
TP78533
TP49524
TP43844
TP40135
TP78664
TP69826
TP59834
TP56420
MRG_38572160
TP8453
TP87928
TP23040
TP5797
TP31991
TP22453
TP56457
TP29084
TP82709
TP12820
TP60638
TP36676
TP80225
TP5601
TP26154
MRG_35320783
TP12385
TP14279
TP48408
TP57224
TP87754
TP3963
TP12515
TP60727
TP73513
TP26686

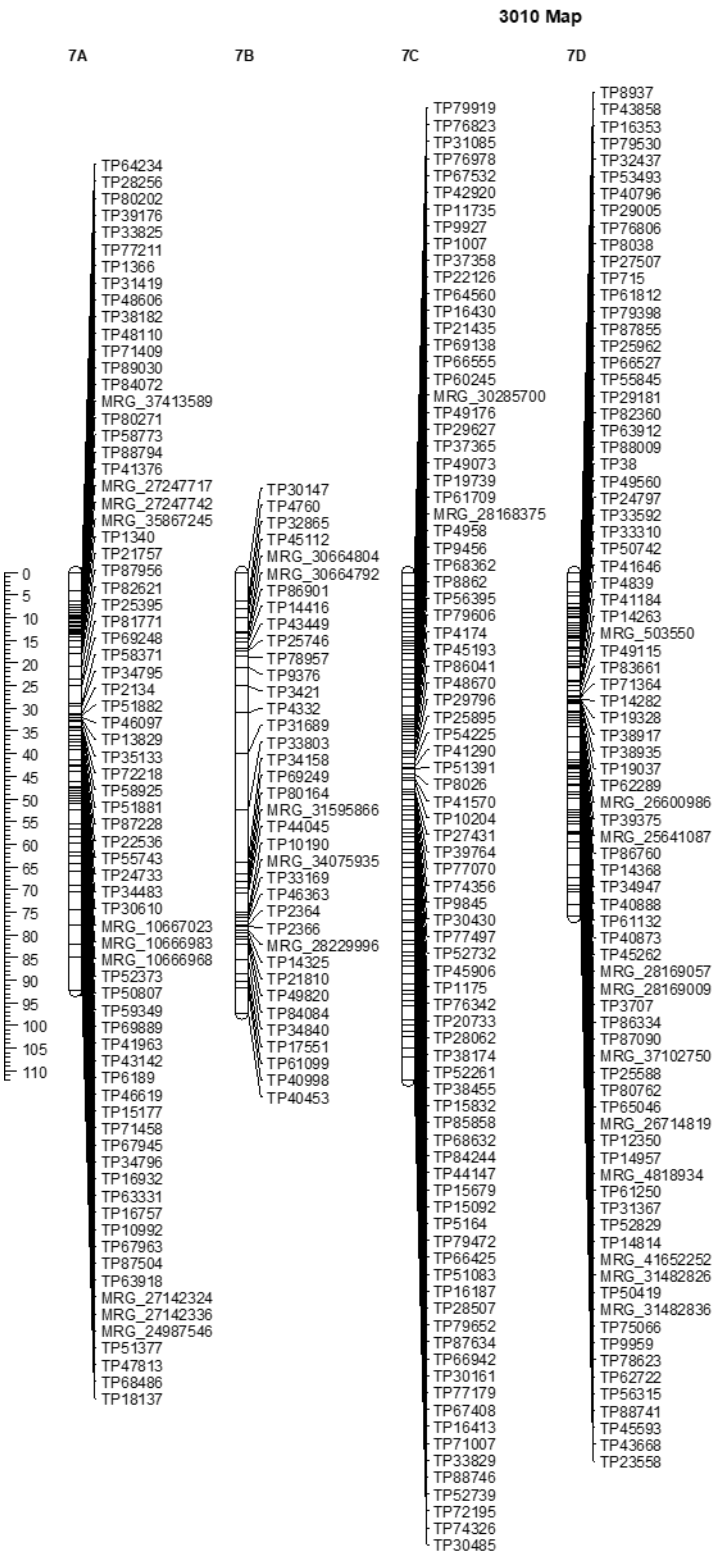

3010 Map
3A
3B
3C
3D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
TP16385
TP32175
TP44970
TP48316
TP32136
TP23378
TP18440
MRG_4754624
MRG_4754683
MRG_6087293
TP32218
TP23752
TP52844
MRG_10610086
TP36716
TP67229
TP80490
TP70924
TP39341
TP60439
TP62030
MRG_38572307 MRG_37063156
TP39863 TP86224
TP1761
MRG_28181310
MRG_16805371
TP14383
TP39827
TP18140
TP59806
TP67013
TP58569
TP88804
TP89465
TP10414
TP3895
TP32327
TP54529
MRG_33239582
TP30031
TP19858
TP10941
TP37432
TP15344
TP26360
TP89244
TP38082
TP52425
TP71671
TP67563
TP76041
TP40960
TP48130
TP22379
TP2592
TP37840
TP59541
TP83334
TP38508
TP68516
TP67008
TP37165
MRG_9104864
TP32765
TP57364
TP48045
TP7707
TP59215
TP36695
TP52130
TP17753
TP34683
TP37707
TP88731
TP76532
TP83110
TP75313
TP45798
TP23666
TP27350
TP8290
TP84356
TP67278
TP70192
TP82034
TP55047
TP71895
TP31602
TP48906
TP60221
TP68861
TP63723
TP46610
TP17460
TP40923
TP71482
TP26680
TP57336
TP59575
TP65852
TP22705
TP41561
TP70195
TP9513
TP634
TP1552
TP13271
TP41487
TP47210
TP49711
MRG_17657401
TP53315
TP89562
TP89597
TP79385
TP56540
TP76561
TP48763
TP86011
TP87583
TP56935
TP5676
TP62551
TP7235
TP45927
TP49015
TP70292
TP3387
TP19043
TP78263
TP49370
TP75378
TP18855
TP71270
TP55943
TP69748
TP43964
TP84552
TP50574
MRG_36069794
TP33172
TP79947
MRG_17830795
MRG_26656909
TP85502
MRG_32256439
TP37583
TP52465
TP72054
TP18964
TP84357
TP46327
TP37337
TP34682
TP38144
TP35334
TP22856
TP63520
TP80485
TP25901
TP86292
TP10177
TP53163
TP62971
TP62402
TP8823
TP64362
TP69697
TP42757
TP67045
TP19939
TP87481
TP32105
TP63037
TP61064
TP67190
TP58690
TP11729
TP79717
TP85520
MRG_22559848
TP30040
TP53864
TP18933
TP66479
TP22877
TP27473
TP88373
TP26775
TP16429
MRG_20846872
MRG_16533874
MRG_16533965
MRG_16533948
TP74002
TP1280
MRG_22245544
TP21496
TP2357
MRG_16284806
TP15269
TP57395
TP26736
TP62582
TP86240
TP59236
TP59153
TP89618
TP81956
TP78693
TP50808
TP43991
TP87713
TP83340
TP70255
TP38681
TP1311
MRG_38527282
MRG_38527400 M
MRG_31477229
MRG_38650252
TP57603
TP16817
TP5092
TP38242
TP14459
TP82209

3010 Map


4A
4B
4C
4D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
TP9123
TP14917
TP57358
TP61731
TP6642
TP14968
TP17692
TP23271
TP10826
TP31525
TP84287
TP21558
TP26871
TP2424
TP34146
TP68230
TP78074
TP89164
TP65472
TP15392
MRG_25685215
TP85237
TP66225
TP62293
TP58768
TP49296
TP64196
TP81727
TP88353
TP33234
MRG_10369239
TP87958
MRG_30257729
TP72111
TP45663
TP65612
MRG_39925469
TP16806
TP58717
TP58652
TP65840
TP27605
TP38904
TP43961
TP16938
TP74499
TP6433
TP58626
TP58969
TP79060
TP64533
TP75584
TP66040
TP77291
TP76073
TP45139
TP64389
TP49020
TP17977
TP8175
TP3619
TP4333
TP76700
TP19920
TP81865
TP1852
TP7329
TP87048
TP10714
TP60313
TP27543
TP82276
TP48125
TP52158
TP30727
TP14262
TP77409
TP73138
TP47789
TP44530
TP65269
TP39453
TP77187
TP61351
TP32407
TP36181
TP11446
TP25366
TP27867
TP7574
TP81237
TP82520
TP41094
TP77943
TP31164
TP23928
TP78015
MRG_28601522
TP36659
TP57924
TP26167
TP57790
TP23017
TP59836
MRG_28210458
TP49265
TP54487
TP52685
TP23120
TP32465
MRG_1128443
TP49337
TP43597
TP65152
TP15305
TP71537
TP34173
TP50014
TP80115
TP29393
TP77953
TP60840
TP24712
TP13564
TP89487
TP86244
MRG_4705953
MRG_11590906
TP66793
TP80397
TP19662
MRG_18042076
TP64526
MRG_8657698
TP13953
TP46820
TP52535
TP6532
TP2323
TP4218
TP35994
TP6857
TP70461
TP38577
TP89211
TP80478
TP81375
TP45699
TP19736
TP80106
TP47289
TP5485
TP14564
TP56665
TP49398
TP81316
TP43293
TP36908
TP60818
TP49984
TP33358
TP85932
TP58826
TP75340
TP45810
TP57107
TP193
TP59910
TP56370
TP68661
TP21971
TP25554
TP89619
TP55689
TP54014
TP56893
TP24046
TP86038
TP23173
TP30941
TP66236
TP55102
TP39710
TP6057
TP55276
TP10534
TP16200
TP72516
TP15380
TP72009
TP7432
TP63385
TP73880
TP74886
TP82278
TP17892
TP47283
TP50060
TP50144
TP21345
TP35410
TP85051
TP82625
TP67038
TP74922
TP63778
TP64539
TP36400
TP61230
TP41553
TP9471
TP31919
TP60513
TP76570
TP5459
TP9221
TP75627
TP73022
TP17725
TP55786
TP48666
TP88532
TP82884
TP68478
TP48214
TP16965
TP2084
TP57786
TP63144
TP35849
TP51170
TP2479
TP77859
TP46263
MRG_28269097
TP3156
TP24127
TP68607
TP58192
TP6349
TP30418
TP88405
TP69094
TP33056
MRG_4464482
MRG_4464574
TP4049
TP43038
TP41829
TP35578
TP32045
TP34525
TP74622
TP83830
TP88593
TP67794
TP37212
MRG_29474256
TP74819
TP1314
TP64785
TP15227
TP84242
TP43613
TP74637
TP3711
TP41970
TP68118
TP45949
TP44386
TP39634
TP18170
TP24479
TP35028
TP31381
TP79021
TP10575
TP17282
TP89146
MRG_30877327
TP61925
TP48720
TP41069
TP74691
TP20211
TP57529
TP65653
TP52076
TP11117
TP37010
TP23172
TP85400
TP59614
TP12402
TP46983
TP16126
TP82585
TP64707
TP61536
TP83938
TP32956
TP55849
TP86910


3010 Map
5A
5B
5C
5D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
TP13010
TP66794
TP20149
TP65005
TP78892
TP43723
TP9239
TP39911
MRG_29270560
TP60791
TP39643
TP26945
TP89325
TP59361
TP19434
TP89078
TP11458
TP60032
MRG_4673463 TP63107
TP51755
TP75069
TP2595
TP46688
TP9305
TP2747
TP78941
TP8230
TP30308
TP76039
MRG_28485316
TP63204
TP78616
TP67077
TP48756
TP60366
TP13788
TP87234
TP56796
TP65086
TP25791
TP7988 TP37228
TP51306
TP79930
TP1845
TP81168
TP86170
TP18826
TP7315
TP1623
TP82148
TP16803
TP4784
TP83935
TP77241
TP38846
TP62566
TP8348
TP46987
TP81767
TP38019
TP54828
TP64873
TP50121
TP34762
TP62133
TP12913
TP15620
TP57295
TP58101
TP29642
TP44743
TP77310
TP23668
TP71245
TP27490
TP74416
TP32698
TP66881
TP67661
TP59715
TP25916
TP35045
TP43026
TP60095
TP53554
TP39445
TP50991
TP53977
TP77297
TP4136
TP13348
TP82736
TP1514
TP79789
TP70426
TP56521
TP68482
MRG_29270539
TP12797
TP14417
TP41037
TP17022
TP6028
TP8196
TP88495
TP50116
TP73620
TP15607
TP36423
TP78538
TP26103
TP33717
TP51604 TP49845
TP61527
TP44290
TP42742
TP61198
TP51007
TP10407
TP34131
TP60294
TP7890
TP33353
TP83890
TP36909
TP24193
TP15194
TP64614
TP75516
TP13006
TP78079
TP88647
TP88207
TP54468
TP48138
MRG_26976586
TP81726
TP39352
TP77766
TP52573
MRG_15346797
MRG_15219648
TP86625
TP49882
MRG_4209212
MRG_4209240
TP75309
TP512
MRG_5308744
TP71157
TP80527
MRG_21568602
MRG_7177936
MRG_6128136
TP1974
TP1277
TP49062
TP47721
TP33496
TP58633
TP51625
TP7532
TP74649
TP29279
TP61569
TP41347
TP71632
MRG_7177893
MRG_38833281
TP58093
MRG_14393781
TP86056
TP59758
TP39164
MRG_5707962
MRG_5707961
MRG_5707960
TP48797
TP62807
TP40587
TP77227
TP19119
TP49006
TP29579
TP62160
TP44672
TP80845
TP66670
TP61232
TP70116
MRG_22254083
TP31552
TP28126
TP71843
TP47467
TP81964
TP51842
TP48237
TP27411
TP4050
TP45390
TP58370
TP87626
TP42064
TP69736
TP9077
TP38263
TP3592


3010 Map
6A
6B
6C
6D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
TP42271
TP2100
TP30032
TP46360
TP21849
TP86156
TP56233
TP47638
TP38258
TP34265
TP16463
TP8233
TP16218
TP85209
TP34006
MRG_20870463
TP82743
TP84177
TP47259
TP50509
TP3440
TP68306
TP62263
TP82318
TP66328
TP81768
TP11690
MRG_29468741
TP87072
TP66339
TP11954
TP65256
TP47046
MRG_29428265
MRG_29428235
TP49922
TP71964
TP59503
TP81621
TP69300
MRG_15694137
MRG_2387265
MRG_9144699
TP81150
TP66143
TP86420
MRG_8375985
TP53454
TP47130
TP33806
TP44890
TP43630
TP41870
TP33012
TP70906
TP8573
TP58667
TP23495
TP47194
TP54512
TP38751
TP47451
TP33340
TP81977
MRG_16533757
TP8588
MRG_26582464
TP56810
TP59233
TP3633
TP45272
TP19122
TP20723
TP62683
TP86842
TP61778
TP73950
TP5629
TP67231
TP25482
TP44520
MRG_26744449
TP25817
TP11778
MRG_4804288
MRG_4804309
MRG_35267404 MRG_1170639
TP88260
TP86241
TP59512
MRG_26874925
TP25991
TP82851
MRG_9630613
TP80984
TP45472
MRG_10731573
TP64260
TP30174
TP34869
TP75733
TP3158
MRG_8657694
TP52946
TP5376
MRG_4038781
MRG_6693677
TP46019
TP71716
TP59053
TP76802
TP49952
MRG_10825492
MRG_32327921
TP9698
TP34669
TP71128
MRG_28382933
MRG_20099263
MRG_20099160
TP646
MRG_28476101
TP73057
TP29142
TP45820
TP13650
TP36879
TP17338
MRG_10618574
TP25048
TP53481
MRG_26680267
MRG_26680303
TP74917
TP77043
TP307
TP52704
TP83895
TP80446
TP19593
TP50371
TP44504
TP36403
TP48315
TP43187
TP87462
TP20467
TP76079
TP14679
TP79776
TP45363
TP47387
TP56891
TP45389
TP62889
MRG_32429735
TP83635
TP89368
TP77110
TP63978
TP25011
TP75307
MRG_26744516
TP48041
TP41942
TP53223
TP6882
TP65002
TP32318
TP58479
TP31578
TP3632
TP27914
TP82498
TP1248
TP2654
TP66762
TP47172
TP13417
TP18824
TP66684
TP86331
MRG_7282984
TP11091
TP8156
MRG_6693691
TP58279
MRG_10617375
TP11326
TP41327
MRG_20870462
TP54474
MRG_20870471
TP80593
MRG_19051799
MRG_20099157
MRG_21416951
TP79359
TP12473
MRG_25638053
TP57727
MRG_26960676
TP74928
MRG_15354456
MRG_15354514
TP77755
TP75093
TP22469
TP17559
TP36663
TP40969
TP81207
TP4357
TP36795
TP10644
TP36118
TP12761
TP8970
TP26367
TP45103
TP46105
TP18739
TP83279
TP16313
MRG_2402742
TP18699
TP49748
MRG_7512674
MRG_9104201
MRG_26202522
MRG_28149596
MRG_21107772
MRG_21107845
MRG_30249033
MRG_30288448
TP72126
TP20622
TP41197
TP22508
TP45805
TP79933
TP26258
TP63620
TP10513
TP71045
TP70625
TP68772
TP62531
TP73743
TP21040
TP25699
TP73707
TP79583
TP44473


3010 Map
7A
7B
7C
7D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
TP64234
TP28256
TP80202
TP39176
TP33825
TP77211
TP1366
TP31419
TP48606
TP38182
TP48110
TP71409
TP89030
TP84072
MRG_37413589
TP80271
TP58773
TP88794
TP41376
MRG_27247717
MRG_27247742
MRG_35867245
TP1340
TP21757
TP87956
TP82621
TP25395
TP81771
TP69248
TP58371
TP34795
TP2134
TP51882
TP46097
TP13829
TP35133
TP72218
TP58925
TP51881
TP87228
TP22536
TP55743
TP24733
TP34483
TP30610
MRG_10667023
MRG_10666983
MRG_10666968
TP52373
TP50807
TP59349
TP69889
TP41963
TP43142
TP6189
TP46619
TP15177
TP71458
TP67945
TP34796
TP16932
TP63331
TP16757
TP10992
TP67963
TP87504
TP63918
MRG_27142324
MRG_27142336
MRG_24987546
TP51377
TP47813
TP68486
TP18137
TP30147
TP4760
TP32865
TP45112
MRG_30664804
MRG_30664792
TP86901
TP14416
TP43449
TP25746
TP78957
TP9376
TP3421
TP4332
TP31689
TP33803
TP34158
TP69249
TP80164
MRG_31595866
TP44045
TP10190
MRG_34075935
TP33169
TP46363
TP2364
TP2366
MRG_28229996
TP14325
TP21810
TP49820
TP84084
TP34840
TP17551
TP61099
TP40998
TP40453
TP79919
TP76823
TP31085
TP76978
TP67532
TP42920
TP11735
TP9927
TP1007
TP37358
TP22126
TP64560
TP16430
TP21435
TP69138
TP66555
TP60245
MRG_30285700
TP49176
TP29627
TP37365
TP49073
TP19739
TP61709
MRG_28168375
TP4958
TP9456
TP68362
TP8862
TP56395
TP79606
TP4174
TP45193
TP86041
TP48670
TP29796
TP25895
TP54225
TP41290
TP51391
TP8026
TP41570
TP10204
TP27431
TP39764
TP77070
TP74356
TP9845
TP30430
TP77497
TP52732
TP45906
TP1175
TP76342
TP20733
TP28062
TP38174
TP52261
TP38455
TP15832
TP85858
TP68632
TP84244
TP44147
TP15679
TP15092
TP5164
TP79472
TP66425
TP51083
TP16187
TP28507
TP79652
TP87634
TP66942
TP30161
TP77179
TP67408
TP16413
TP71007
TP33829
TP88746
TP52739
TP72195
TP74326
TP30485
TP8937
TP43858
TP16353
TP79530
TP32437
TP53493
TP40796
TP29005
TP76806
TP8038
TP27507
TP715
TP61812
TP79398
TP87855
TP25962
TP66527
TP55845
TP29181
TP82360
TP63912
TP88009
TP38
TP49560
TP24797
TP33592
TP33310
TP50742
TP41646
TP4839
TP41184
TP14263
MRG_503550
TP49115
TP83661
TP71364
TP14282
TP19328
TP38917
TP38935
TP19037
TP62289
MRG_26600986
TP39375
MRG_25641087
TP86760
TP14368
TP34947
TP40888
TP61132
TP40873
TP45262
MRG_28169057
MRG_28169009
TP3707
TP86334
TP87090
MRG_37102750
TP25588
TP80762
TP65046
MRG_26714819
TP12350
TP14957
MRG_4818934
TP61250
TP31367
TP52829
TP14814
MRG_41652252
MRG_31482826
TP50419
MRG_31482836
TP75066
TP9959
TP78623
TP62722
TP56315
TP88741
TP45593
TP43668
TP23558

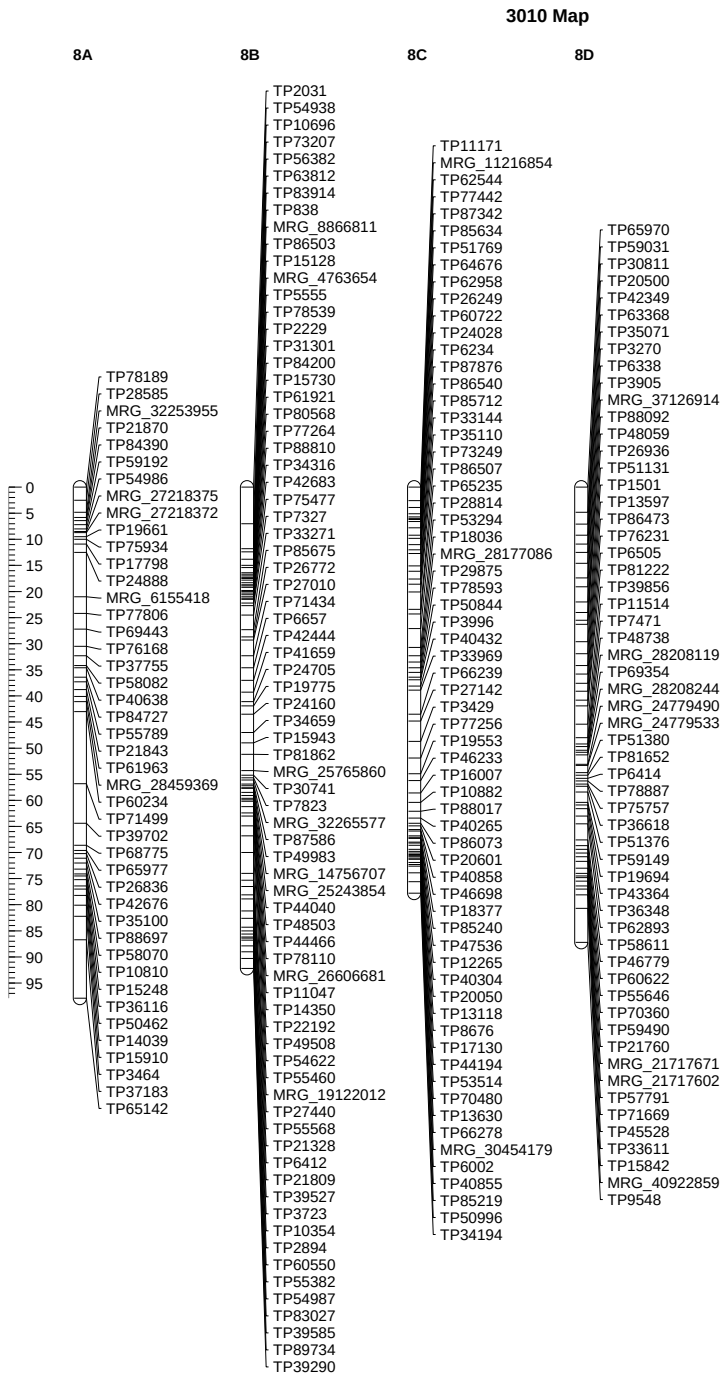
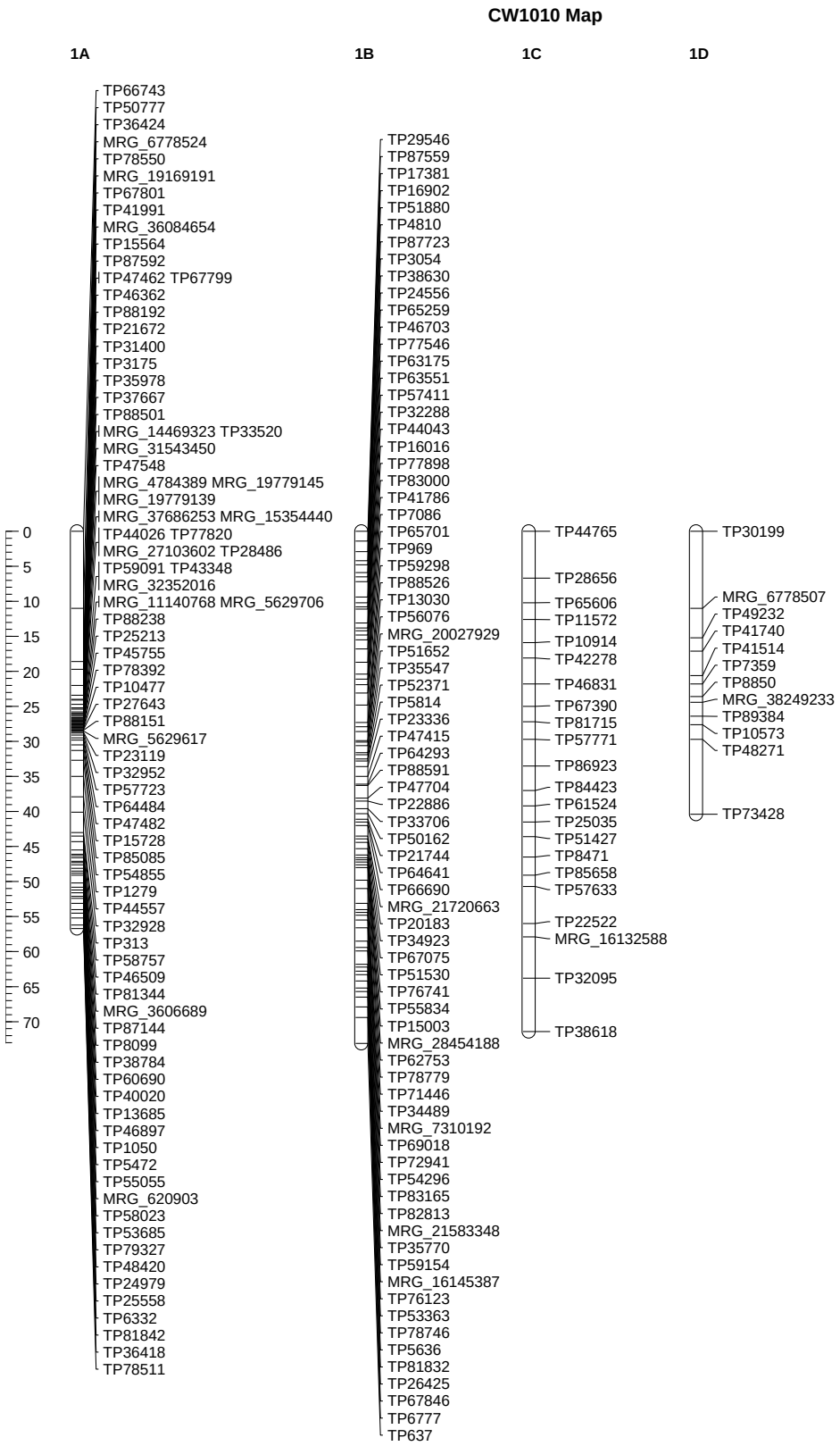
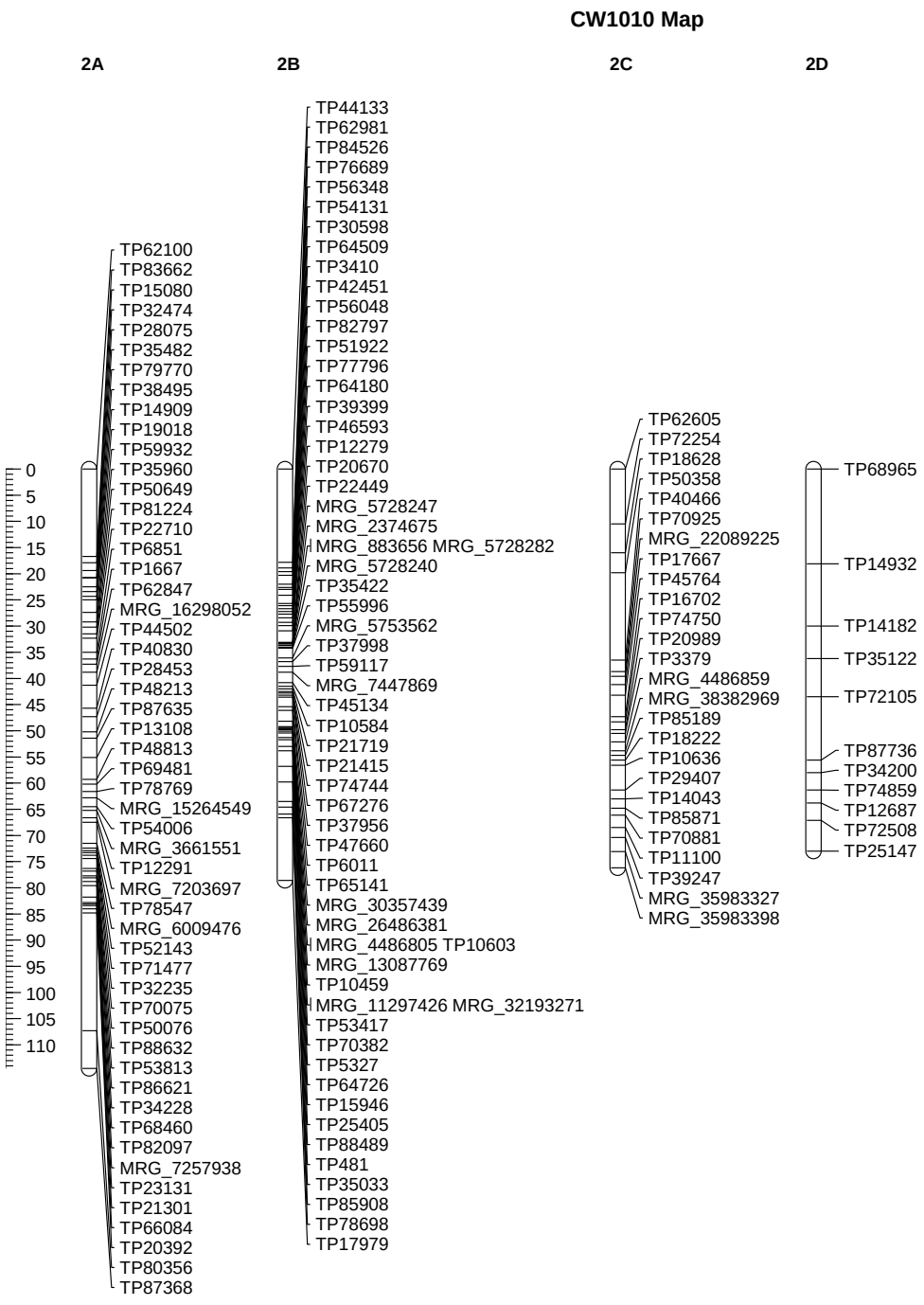
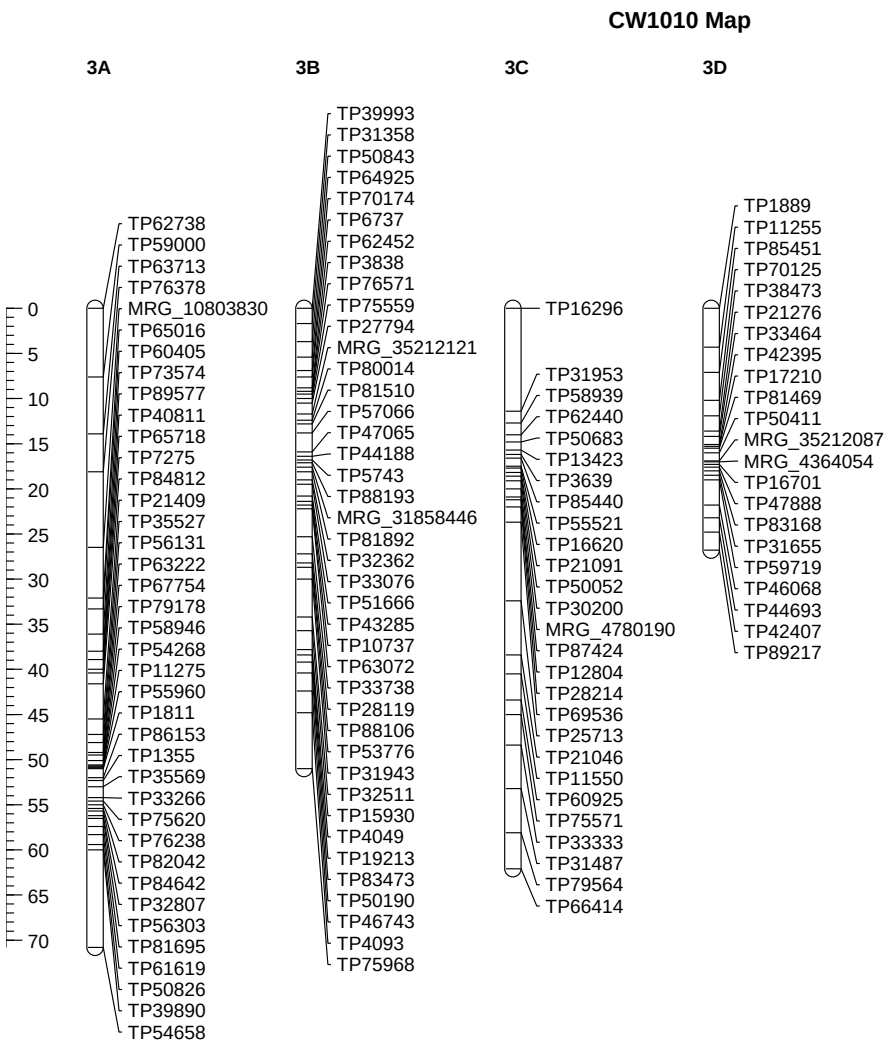
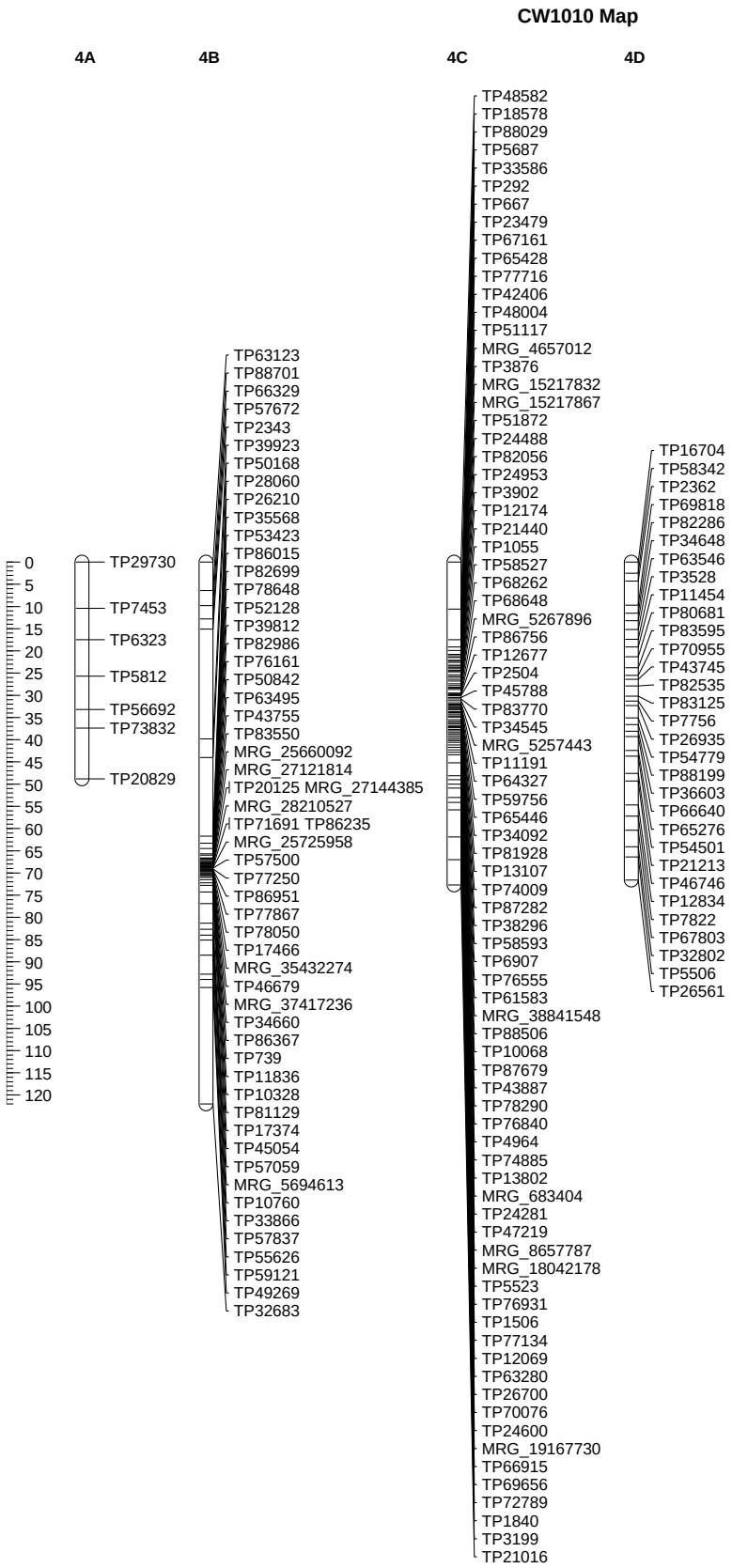


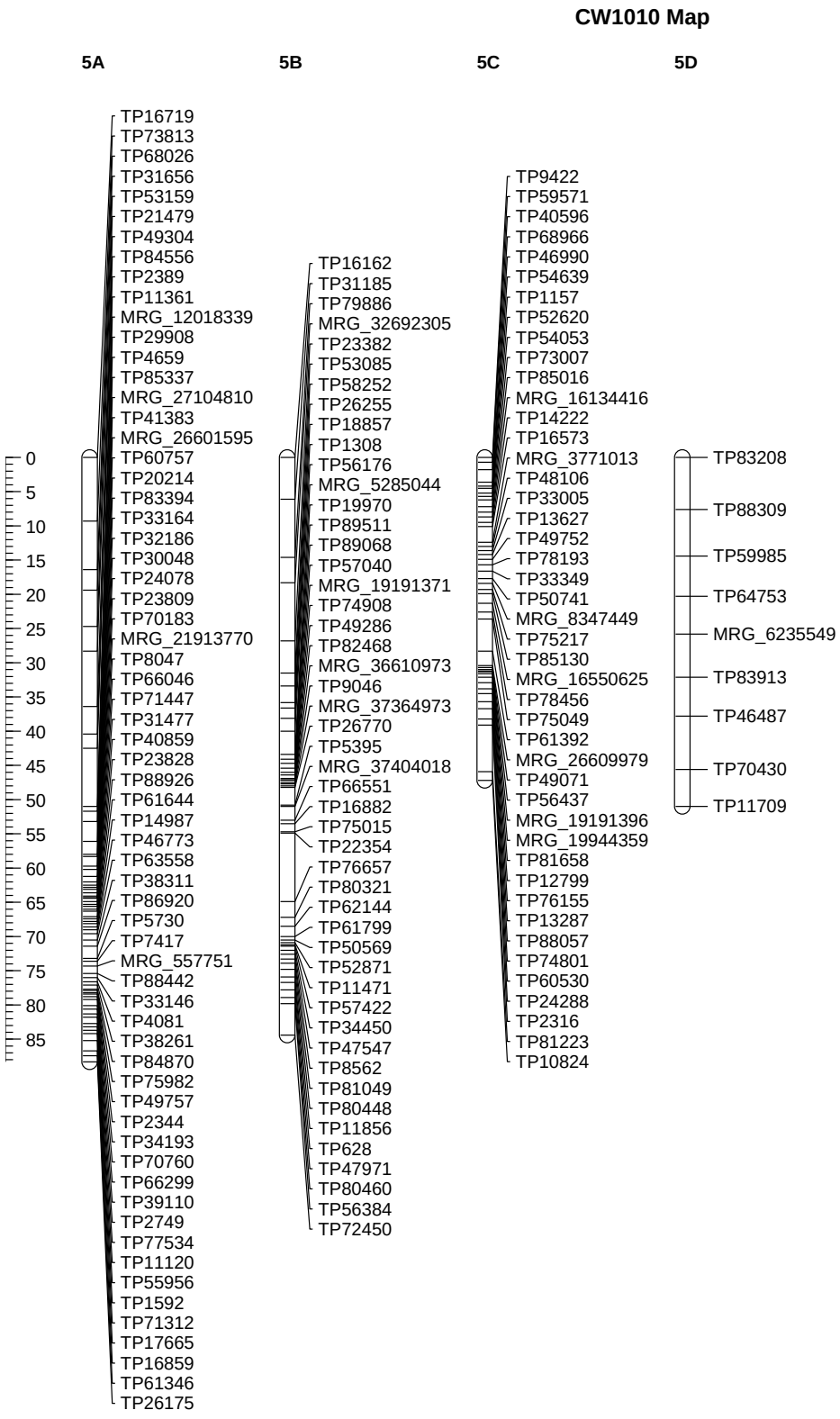
Figure 2.S1. Thirty-two linkage groups for the maternal parent 3010 map. The positions of SNPs were given in Kosambi centimorgan (cM). Four haplotype maps (A, B, C, D) were grouped per chromosome based on the positions of SNPs obtained from BLAST analysis using *M. truncatula* reference genome.

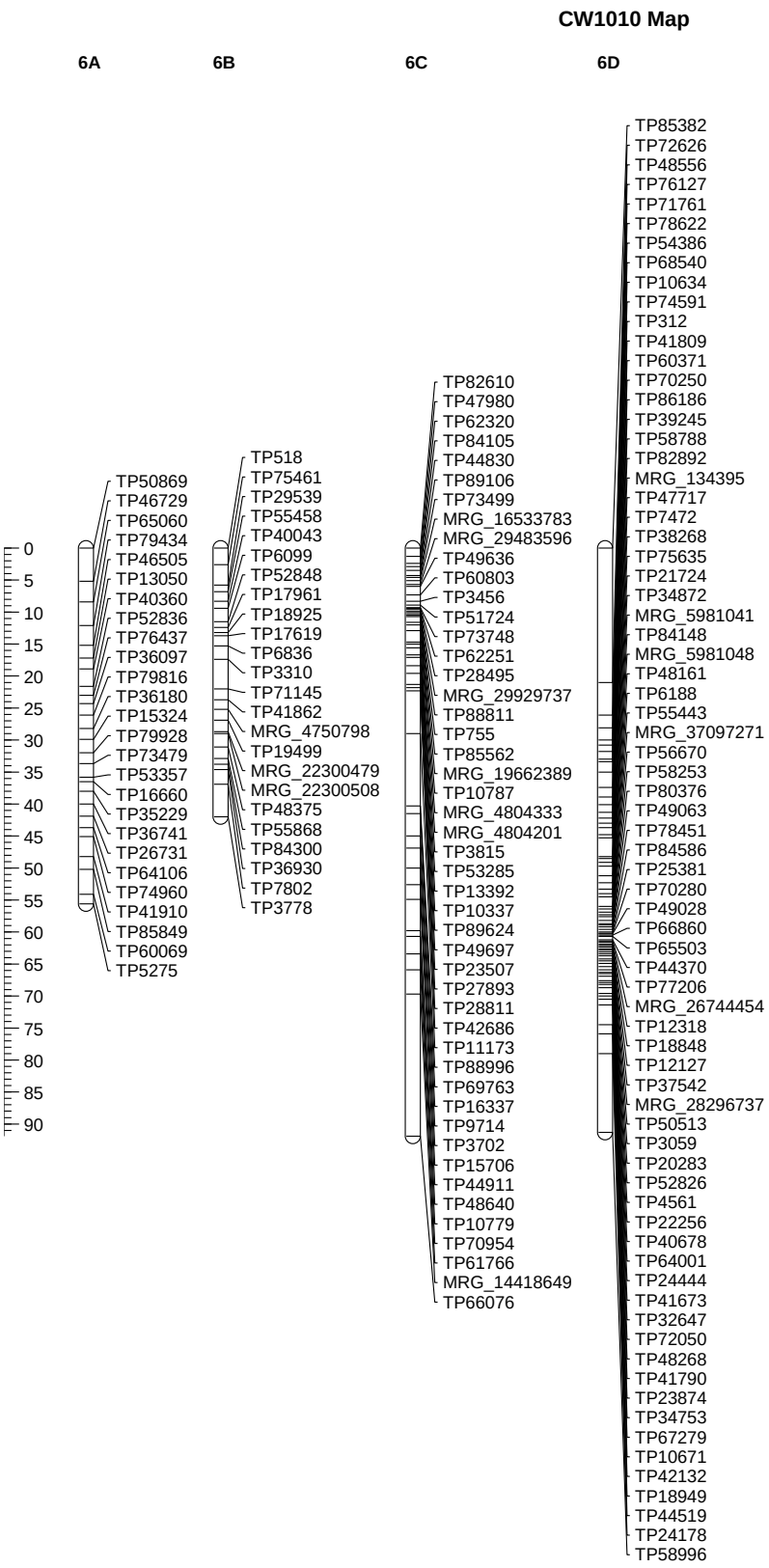

CW1010 Map
1A
1B
1C
1D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TP66743
TP50777
TP36424
MRG_6778524
TP78550
MRG_19169191
TP67801
TP41991
MRG_36084654
TP15564
TP87592
TP47462 TP67799
TP46362
TP88192
TP21672
TP31400
TP3175
TP35978
TP37667
TP88501
MRG_14469323 TP33520
MRG_31543450
TP47548
MRG_4784389 MRG_19779145
MRG_19779139
MRG_37686253 MRG_15354440
TP44026 TP77820
MRG_27103602 TP28486
TP59091 TP43348
MRG_32352016
MRG_11140768 MRG_5629706
TP88238
TP25213
TP45755
TP78392
TP10477
TP27643
TP88151
MRG_5629617
TP23119
TP32952
TP57723
TP64484
TP47482
TP15728
TP85085
TP54855
TP1279
TP44557
TP32928
TP313
TP58757
TP46509
TP81344
MRG_3606689
TP87144
TP8099
TP38784
TP60690
TP40020
TP13685
TP46897
TP1050
TP5472
TP55055
MRG_620903
TP58023
TP53685
TP79327
TP48420
TP24979
TP25558
TP6332
TP81842
TP36418
TP78511
TP29546
TP87559
TP17381
TP16902
TP51880
TP4810
TP87723
TP3054
TP38630
TP24556
TP65259
TP46703
TP77546
TP63175
TP63551
TP57411
TP32288
TP44043
TP16016
TP77898
TP83000
TP41786
TP7086
TP65701
TP969
TP59298
TP88526
TP13030
TP56076
MRG_20027929
TP51652
TP35547
TP52371
TP5814
TP23336
TP47415
TP64293
TP88591
TP47704
TP22886
TP33706
TP50162
TP21744
TP64641
TP66690
MRG_21720663
TP20183
TP34923
TP67075
TP51530
TP76741
TP55834
TP15003
MRG_28454188
TP62753
TP78779
TP71446
TP34489
MRG_7310192
TP69018
TP72941
TP54296
TP83165
TP82813
MRG_21583348
TP35770
TP59154
MRG_16145387
TP76123
TP53363
TP78746
TP5636
TP81832
TP26425
TP67846
TP6777
TP637
TP44765
TP28656
TP65606
TP11572
TP10914
TP42278
TP46831
TP67390
TP81715
TP57771
TP86923
TP84423
TP61524
TP25035
TP51427
TP8471
TP85658
TP57633
TP22522
MRG_16132588
TP32095
TP38618
TP30199
MRG_6778507
TP49232
TP41740
TP41514
TP7359
TP8850
MRG_38249233
TP89384
TP10573
TP48271
TP73428

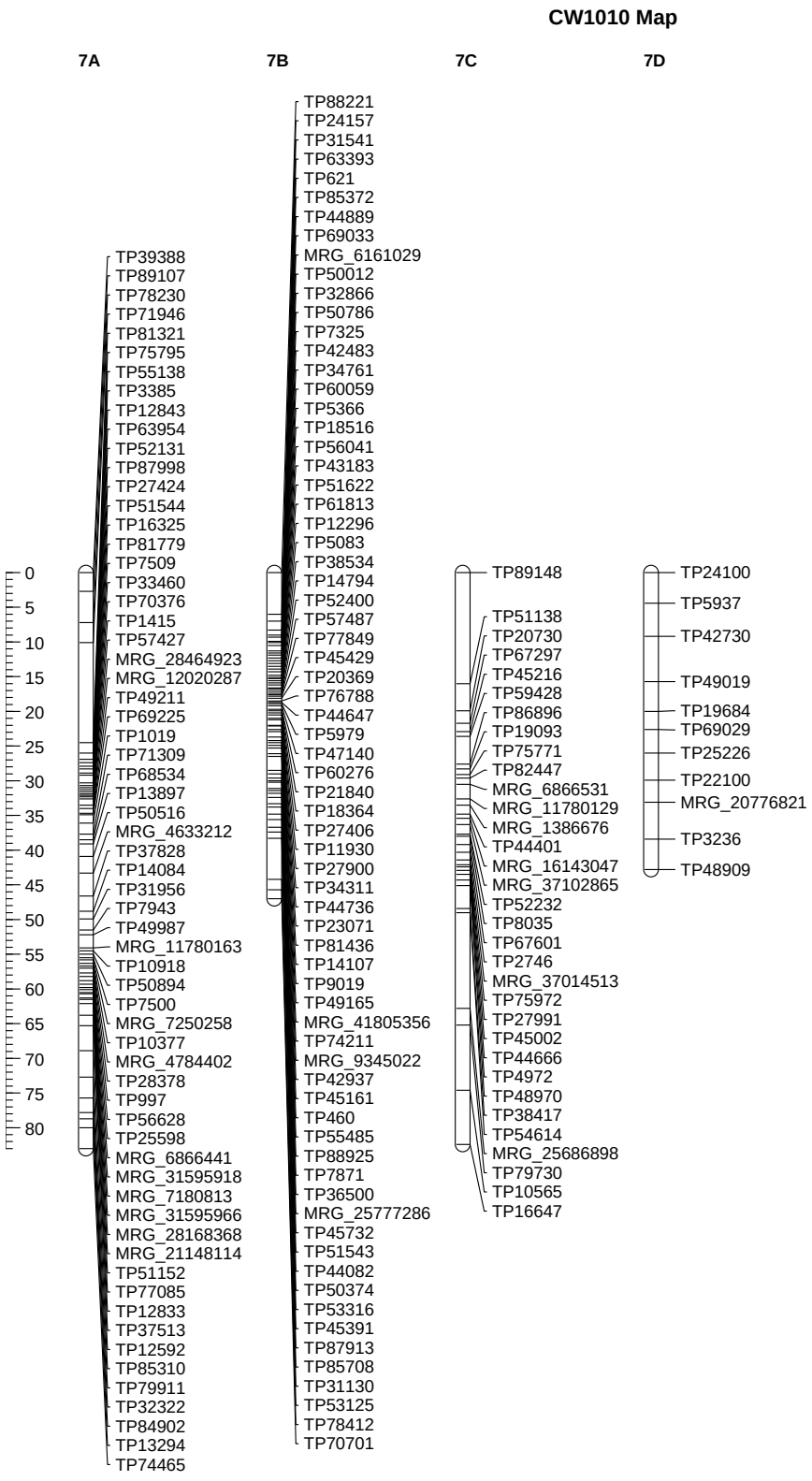

CW1010 Map
2A
2B
2C
2D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
TP62100
TP83662
TP15080
TP32474
TP28075
TP35482
TP79770
TP38495
TP14909
TP19018
TP59932
TP35960
TP50649
TP81224
TP22710
TP6851
TP1667
TP62847
MRG_16298052
TP44502
TP40830
TP28453
TP48213
TP87635
TP13108
TP48813
TP69481
TP78769
MRG_15264549
TP54006
MRG_3661551
TP12291
MRG_7203697
TP78547
MRG_6009476
TP52143
TP71477
TP32235
TP70075
TP50076
TP88632
TP53813
TP86621
TP34228
TP68460
TP82097
MRG_7257938
TP23131
TP21301
TP66084
TP20392
TP80356
TP87368
TP44133
TP62981
TP84526
TP76689
TP56348
TP54131
TP30598
TP64509
TP3410
TP42451
TP56048
TP82797
TP51922
TP77796
TP64180
TP39399
TP46593
TP12279
TP20670
TP22449
MRG_5728247
MRG_2374675
MRG_883656 MRG_5728282
MRG_5728240
TP35422
TP55996
MRG_5753562
TP37998
TP59117
MRG_7447869
TP45134
TP10584
TP21719
TP21415
TP74744
TP67276
TP37956
TP47660
TP6011
TP65141
MRG_30357439
MRG_26486381
MRG_4486805 TP10603
MRG_13087769
TP10459
MRG_11297426 MRG_32193271
TP53417
TP70382
TP5327
TP64726
TP15946
TP25405
TP88489
TP481
TP35033
TP85908
TP78698
TP17979
TP62605
TP72254
TP18628
TP50358
TP40466
TP70925
MRG_22089225
TP17667
TP45764
TP16702
TP74750
TP20989
TP3379
MRG_4486859
MRG_38382969
TP85189
TP18222
TP10636
TP29407
TP14043
TP85871
TP70881
TP11100
TP39247
MRG_35983327
MRG_35983398
TP68965
TP14932
TP14182
TP35122
TP72105
TP87736
TP34200
TP74859
TP12687
TP72508
TP25147


CW1010 Map
3A
3B
3C
3D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TP62738
TP59000
TP63713
TP76378
MRG_10803830
TP65016
TP60405
TP73574
TP89577
TP40811
TP65718
TP7275
TP84812
TP21409
TP35527
TP56131
TP63222
TP67754
TP79178
TP58946
TP54268
TP11275
TP55960
TP1811
TP86153
TP1355
TP35569
TP33266
TP75620
TP76238
TP82042
TP84642
TP32807
TP56303
TP81695
TP61619
TP50826
TP39890
TP54658
TP39993
TP31358
TP50843
TP64925
TP70174
TP6737
TP62452
TP3838
TP76571
TP75559
TP27794
MRG_35212121
TP80014
TP81510
TP57066
TP47065
TP44188
TP5743
TP88193
MRG_31858446
TP81892
TP32362
TP33076
TP51666
TP43285
TP10737
TP63072
TP33738
TP28119
TP88106
TP53776
TP31943
TP32511
TP15930
TP4049
TP19213
TP83473
TP50190
TP46743
TP4093
TP75968
TP16296
TP31953
TP58939
TP62440
TP50683
TP13423
TP3639
TP85440
TP55521
TP16620
TP21091
TP50052
TP30200
MRG_4780190
TP87424
TP12804
TP28214
TP69536
TP25713
TP21046
TP11550
TP60925
TP75571
TP33333
TP31487
TP79564
TP66414
TP1889
TP11255
TP85451
TP70125
TP38473
TP21276
TP33464
TP42395
TP17210
TP81469
TP50411
MRG_35212087
MRG_4364054
TP16701
TP47888
TP83168
TP31655
TP59719
TP46068
TP44693
TP42407
TP89217


CW1010 Map
4A
4B
4C
4D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
TP29730
TP7453
TP6323
TP5812
TP56692
TP73832
TP20829
TP63123
TP88701
TP66329
TP57672
TP2343
TP39923
TP50168
TP28060
TP26210
TP35568
TP53423
TP86015
TP82699
TP78648
TP52128
TP39812
TP82986
TP76161
TP50842
TP63495
TP43755
TP83550
MRG_25660092
MRG_27121814
TP20125 MRG_27144385
MRG_28210527
TP71691 TP86235
MRG_25725958
TP57500
TP77250
TP86951
TP77867
TP78050
TP17466
MRG_35432274
TP46679
MRG_37417236
TP34660
TP86367
TP739
TP11836
TP10328
TP81129
TP17374
TP45054
TP57059
MRG_5694613
TP10760
TP33866
TP57837
TP55626
TP59121
TP49269
TP32683
TP48582
TP18578
TP88029
TP5687
TP33586
TP292
TP667
TP23479
TP67161
TP65428
TP77716
TP42406
TP48004
TP51117
MRG_4657012
TP3876
MRG_15217832
MRG_15217867
TP51872
TP24488
TP82056
TP24953
TP3902
TP12174
TP21440
TP1055
TP58527
TP68262
TP68648
MRG_5267896
TP86756
TP12677
TP2504
TP45788
TP83770
TP34545
MRG_5257443
TP11191
TP64327
TP59756
TP65446
TP34092
TP81928
TP13107
TP74009
TP87282
TP38296
TP58593
TP6907
TP76555
TP61583
MRG_38841548
TP88506
TP10068
TP87679
TP43887
TP78290
TP76840
TP4964
TP74885
TP13802
MRG_683404
TP24281
TP47219
MRG_8657787
MRG_18042178
TP5523
TP76931
TP1506
TP77134
TP12069
TP63280
TP26700
TP70076
TP24600
MRG_19167730
TP66915
TP69656
TP72789
TP1840
TP3199
TP21016
TP16704
TP58342
TP2362
TP69818
TP82286
TP34648
TP63546
TP3528
TP11454
TP80681
TP83595
TP70955
TP43745
TP82535
TP83125
TP7756
TP26935
TP54779
TP88199
TP36603
TP66640
TP65276
TP54501
TP21213
TP46746
TP12834
TP7822
TP67803
TP32802
TP5506
TP26561


CW1010 Map
5A
5B
5C
5D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
TP16719
TP73813
TP68026
TP31656
TP53159
TP21479
TP49304
TP84556
TP2389
TP11361
MRG_12018339
TP29908
TP4659
TP85337
MRG_27104810
TP41383
MRG_26601595
TP60757
TP20214
TP83394
TP33164
TP32186
TP30048
TP24078
TP23809
TP70183
MRG_21913770
TP8047
TP66046
TP71447
TP31477
TP40859
TP23828
TP88926
TP61644
TP14987
TP46773
TP63558
TP38311
TP86920
TP5730
TP7417
MRG_557751
TP88442
TP33146
TP4081
TP38261
TP84870
TP75982
TP49757
TP2344
TP34193
TP70760
TP66299
TP39110
TP2749
TP77534
TP11120
TP55956
TP1592
TP71312
TP17665
TP16859
TP61346
TP26175
TP16162
TP31185
TP79886
MRG_32692305
TP23382
TP53085
TP58252
TP26255
TP18857
TP1308
TP56176
MRG_5285044
TP19970
TP89511
TP89068
TP57040
MRG_19191371
TP74908
TP49286
TP82468
MRG_36610973
TP9046
MRG_37364973
TP26770
TP5395
MRG_37404018
TP66551
TP16882
TP75015
TP22354
TP76657
TP80321
TP62144
TP61799
TP50569
TP52871
TP11471
TP57422
TP34450
TP47547
TP8562
TP81049
TP80448
TP11856
TP628
TP47971
TP80460
TP56384
TP72450
TP9422
TP59571
TP40596
TP68966
TP46990
TP54639
TP1157
TP52620
TP54053
TP73007
TP85016
MRG_16134416
TP14222
TP16573
MRG_3771013
TP48106
TP33005
TP13627
TP49752
TP78193
TP33349
TP50741
MRG_8347449
TP75217
TP85130
MRG_16550625
TP78456
TP75049
TP61392
MRG_26609979
TP49071
TP56437
MRG_19191396
MRG_19944359
TP81658
TP12799
TP76155
TP13287
TP88057
TP74801
TP60530
TP24288
TP2316
TP81223
TP10824
TP83208
TP88309
TP59985
TP64753
MRG_6235549
TP83913
TP46487
TP70430
TP11709


CW1010 Map
6A
6B
6C
6D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
TP50869
TP46729
TP65060
TP79434
TP46505
TP13050
TP40360
TP52836
TP76437
TP36097
TP79816
TP36180
TP15324
TP79928
TP73479
TP53357
TP16660
TP35229
TP36741
TP26731
TP64106
TP74960
TP41910
TP85849
TP60069
TP5275
TP518
TP75461
TP29539
TP55458
TP40043
TP6099
TP52848
TP17961
TP18925
TP17619
TP6836
TP3310
TP71145
TP41862
MRG_4750798
TP19499
MRG_22300479
MRG_22300508
TP48375
TP55868
TP84300
TP36930
TP7802
TP3778
TP82610
TP47980
TP62320
TP84105
TP44830
TP89106
TP73499
MRG_16533783
MRG_29483596
TP49636
TP60803
TP3456
TP51724
TP73748
TP62251
TP28495
MRG_29929737
TP88811
TP755
TP85562
MRG_19662389
TP10787
MRG_4804333
MRG_4804201
TP3815
TP53285
TP13392
TP10337
TP89624
TP49697
TP23507
TP27893
TP28811
TP42686
TP11173
TP88996
TP69763
TP16337
TP9714
TP3702
TP15706
TP44911
TP48640
TP10779
TP70954
TP61766
MRG_14418649
TP66076
TP85382
TP72626
TP48556
TP76127
TP71761
TP78622
TP54386
TP68540
TP10634
TP74591
TP312
TP41809
TP60371
TP70250
TP86186
TP39245
TP58788
TP82892
MRG_134395
TP47717
TP7472
TP38268
TP75635
TP21724
TP34872
MRG_5981041
TP84148
MRG_5981048
TP48161
TP6188
TP55443
MRG_37097271
TP56670
TP58253
TP80376
TP49063
TP78451
TP84586
TP25381
TP70280
TP49028
TP66860
TP65503
TP44370
TP77206
MRG_26744454
TP12318
TP18848
TP12127
TP37542
MRG_28296737
TP50513
TP3059
TP20283
TP52826
TP4561
TP22256
TP40678
TP64001
TP24444
TP41673
TP32647
TP72050
TP48268
TP41790
TP23874
TP34753
TP67279
TP10671
TP42132
TP18949
TP44519
TP24178
TP58996


CW1010 Map
7A
7B
7C
7D
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
TP39388
TP89107
TP78230
TP71946
TP81321
TP75795
TP55138
TP3385
TP12843
TP63954
TP52131
TP87998
TP27424
TP51544
TP16325
TP81779
TP7509
TP33460
TP70376
TP1415
TP57427
MRG_28464923
MRG_12020287
TP49211
TP69225
TP1019
TP71309
TP68534
TP13897
TP50516
MRG_4633212
TP37828
TP14084
TP31956
TP7943
TP49987
MRG_11780163
TP10918
TP50894
TP7500
MRG_7250258
TP10377
MRG_4784402
TP28378
TP997
TP56628
TP25598
MRG_6866441
MRG_31595918
MRG_7180813
MRG_31595966
MRG_28168368
MRG_21148114
TP51152
TP77085
TP12833
TP37513
TP12592
TP85310
TP79911
TP32322
TP84902
TP13294
TP74465
TP88221
TP24157
TP31541
TP63393
TP621
TP85372
TP44889
TP69033
MRG_6161029
TP50012
TP32866
TP50786
TP7325
TP42483
TP34761
TP60059
TP5366
TP18516
TP56041
TP43183
TP51622
TP61813
TP12296
TP5083
TP38534
TP14794
TP52400
TP57487
TP77849
TP45429
TP20369
TP76788
TP44647
TP5979
TP47140
TP60276
TP21840
TP18364
TP27406
TP11930
TP27900
TP34311
TP44736
TP23071
TP81436
TP14107
TP9019
TP49165
MRG_41805356
TP74211
MRG_9345022
TP42937
TP45161
TP460
TP55485
TP88925
TP7871
TP36500
MRG_25777286
TP45732
TP51543
TP44082
TP50374
TP53316
TP45391
TP87913
TP85708
TP31130
TP53125
TP78412
TP70701
TP89148
TP51138
TP20730
TP67297
TP45216
TP59428
TP86896
TP19093
TP75771
TP82447
MRG_6866531
MRG_11780129
MRG_1386676
TP44401
MRG_16143047
MRG_37102865
TP52232
TP8035
TP67601
TP2746
MRG_37014513
TP75972
TP27991
TP45002
TP44666
TP4972
TP48970
TP38417
TP54614
MRG_25686898
TP79730
TP10565
TP16647
TP24100
TP5937
TP42730
TP49019
TP19684
TP69029
TP25226
TP22100
MRG_20776821
TP3236
TP48909

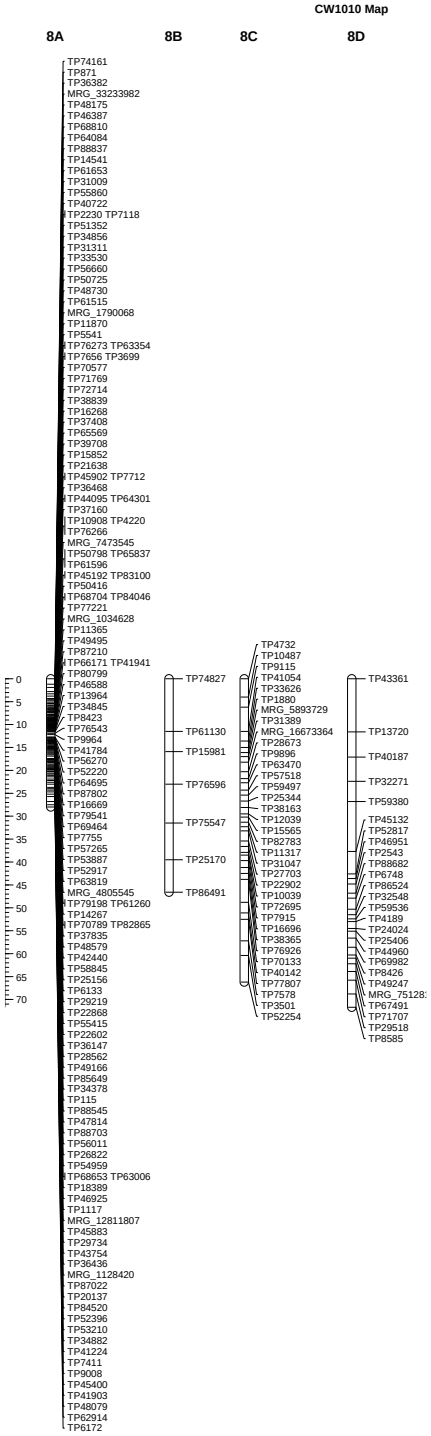


Figure 2.S2. Thirty-two linkage groups for the paternal parent CW 1010 map. The positions of SNPs were given in Kosambi centimorgan (cM). Four haplotype maps (A, B, C, D) were grouped per chromosome based on the positions of SNPs obtained from BLAST analysis using *M. truncatula* reference genome.

CHAPTER 3

DISSECTING GENOTYPIC AND PHENOTYPIC VARIATIONS IN SPRING FLOWERING TIME AND BIOMASS YIELD IN ALFALFA

Adhikari L, Makaju SO, Missaoui AM (2018). To be submitted to *G3:Genes, Genomes, Genetics*

Abstract

The genetic and genomic basis of flowering time and biomass yield in alfalfa (*Medicago sativa* L.) remain poorly understood mainly due to the polyploidy nature of the species and the lack of genomic resources. However, NGS methods such as Genotyping-by-Sequencing (GBS) has made possible the generation of thousands to millions of SNP markers for high-throughput genotyping and genetic analysis in species without reference genomes. We used linkage maps constructed using GBS SNPs, based on single dose allele (SDA) markers, to map timing of flowering (TOF), spring yield (SY) and cumulative summer biomass (CSB). An F1 mapping population was developed by hand pollination of the cultivars 3010 (♀) and CW 1010 (♂). The parents 3010 and CW 1010 had contrasting fall dormancy (FD) and winter hardiness (WH). The F1 progenies, parents, and checks were planted in a randomized complete block design (RCBD) with three replications at Watkinsville (JPC Farm) and Blairsville, GA. The TOF was estimated using Julian calendar days. The biomass of mapping population was harvested mechanically for SY and CSB. Phenotypic correlations between the various traits were estimated. Phenotypic value of traits was estimated using the least square means with Proc GLM. QTL mapping was performed using the composite interval mapping method in WinQTL Cartographer (2.5) and the QTLs were declared with LOD threshold ≥ 3.0 in each parental linkage map. Phenotypic correlations between TOF and fall dormancy rating (FDR) in alfalfa were moderate. The non-dormant F1 plants flowered earlier in spring and the correlation coefficient between TOF and FDR was up to ($r = -0.44$, $P < 0.01$). Similarly, the more winter-hardy F1 alfalfa plants flowered later in the spring. The relationship between flowering time and alfalfa biomass yield was not strong. A total of 25 QTLs for TOF, 17 for SY and six QTLs for CSB were detected. Three TOF related QTLs were deemed stable. Some TOF QTLs of alfalfa were in the same region relative to flowering QTLs of *M. truncatula* on chromosome 7 identified previously. Several

putative homologous genes for the SNP sequences of QTL peaks and flanking regions were observed for all three traits. The trait associated SNPs we detected in this study would be valuable for alfalfa improvement via marker-assisted selection (MAS).

Abbreviations

FD = Fall dormancy
WH = Winter hardiness
WS = Winter survival
TOF = Timing of flowering
SY = Spring yield
CSB = Cumulative summer biomass
MAS = Marker-assisted Selection
GBS = Genotyping-by-sequencing
SDA = Single dose allele
AGDD = Accumulated growing degree days

Introduction

Alfalfa (*Medicago sativa* L.), a cool season perennial legume grown for forage, is an autotetraploid (2n=4x = 32), entomophilous and allogamous species [1]. As an excellent source of hay, pasture and silage, alfalfa worldwide acreage is over 30 million ha [2]. Utilizing alfalfa stems as a potential source of feedstock for bioenergy has also been explored [3]. In the US, alfalfa is cultivated in almost all states as a cattle feed and export cash crop, and ranks fourth nationally after corn, soybean and wheat in term of acreages [4]. Alfalfa has also been recognized as an important part of sustainable cropping systems for its deep rooting system, reducing soil erosion and its ability to fix nitrogen. It is an excellent forage source for filling seasonal forage gaps in late fall and early spring that exist because of non-active growth of warm-season forages [5]. Understanding the phenotypic and genotypic relationship of the important agronomic traits is crucial for their simultaneous improvement or for the trade-off balance between these traits. Recently, scientists have realized that simultaneous improvement of both fall dormancy (FD) and winter hardiness (WH) in

alfalfa is possible because the two traits are inherited independently [6-8]. Traits such as, biomass yield, persistence, forage quality, abiotic and biotic stress tolerance, fall dormancy, and flowering time are correlated traits of alfalfa whose breeding and improvement require knowledge of genetics and genomics.

In alfalfa, time of flowering (TOF) is important for completion of reproduction and adaptation to the environment [9]. Alfalfa TOF also serves as a guide for harvesting time as farmers often cut alfalfa at the early bloom stage [10]. Harvesting at the proper stage helps balancing forage quality, yield, and maintaining healthy stubbles for the future stand [11]. After the first cutting, next blooming stage comes in about 28-35 days [10]. Unlike some other forages, harvesting before reaching the full seed stage is common in alfalfa for high total digestible nutrients (TDN) which decreases with maturity stage. Early vs. late flowering are two conditions of concern to a breeder while working on flowering date. In unfavorable climates where drought or heat stresses are major concerns for crop production, early flowering could be a desirable trait because of the short growing season [12]. Genotypes that flower earlier in spring could be a valuable source for filling a seasonal forage gap that exists due to winter low temperature. On the other hand, delayed flowering can also be a desirable trait to minimize damage from abiotic stresses as well as to enhance biomass yield via longer vegetative growth [13]. For instance, in bioenergy crops, such as switchgrass and elephant grass, late flowering enhances yield. Similarly, delayed flowering in alfalfa could be a desirable trait for protecting plants from late winter frost and enhanced biomass. The flower bud initiation to seed pod formation stages in alfalfa are largely environmentally dependent. Photoperiod and temperature have great impacts on alfalfa flowering time [14] while the underlying genetic factors are important to manipulate this trait.

The genetic and genomic basis of flowering time have been investigated extensively in cereals and row crops, whereas such information is scarce in herbaceous perennials. QTLs and candidate genes controlling TOF were reported in several plant species, such as *Arabidopsis* [15], wheat [16], rice [17], and maize [18]. In *Arabidopsis*, three distinct genetic pathways; long-day, autonomous, and gibberellins were reported for flowering time control [19]. Vernalization was also reported as a mechanism associated with flowering time in *Arabidopsis*. By far, legumes such as pea (*Pisum sativum*), soybean (*Glycine max* L.) and *Medicago* have been studied for the genetic basis of variation in TOF. Pierre et al. (2008), found QTLs for flowering date in three mapping populations of *Medicago* on chromosome seven [9]. Further, the FT family florigen (MtFTa1, MtFTb1 and MtFTc) detected for *Medicago* flowering trait successfully complemented the late-flowering Arabidopsis *ft-1* mutant plants and induced early flowering [20]. Similarly, involvement of some genes: MsLFY [21], CONSTANS-LIKE [22], SPL13 [23], MsZFN [24] and MsFRI-L [25] of alfalfa for flowering time variation have been described using reverse genetics approaches such as molecular cloning and gene expression. Nevertheless, single gene expression analysis with a knockout or transgenics seems insufficient to account for the extensive variation in the population [26]. The phenotypic variation present in a population for a quantitative trait such as flowering time can be explained using QTL mapping, where a high-resolution genetic map is available.

High biomass yield is one of the main deciding factors for market acceptance of a newly developed alfalfa cultivar. For a cool season crop like alfalfa, biomass yield after spring regrowth and in subsequent harvests account for a remarkable portion of the year-round production. However, spring biomass yield of alfalfa correlates with other characteristics such as FD [27]. Compared to dormant genotypes, non-dormant accessions often start regrowth earlier in spring, flower earlier, generate high biomass, and start shoot regrowth quicker after harvesting even in subsequent summer

months [27]. Faster spring growth is also a positive indicator of higher summer growth [28]. QTL mapping work for alfalfa using traditional markers and phenotypic data based on plant vigor, height, canopy-width and canopy-density enabled the detection of some forage biomass related QTLs [29]. Some QTLs having phenotypic effects up to 6% and associated with alfalfa biomass in drought stress conditions have been reported [30]. Li et al. (2011) performed genome-wide association (GWAS) mapping using simple sequence repeat (SSR) markers in an alfalfa breeding population and detected markers associated with yield [31]. Another GWAS study reported SNPs associated with biomass yield of a diploid alfalfa (*M. falcata*) population in the regions of *Medicago* genes for early growth, meristematic development and cell growth/division [32]. Similarly, Li et al. (2015) conducted genomic selection (GS) in alfalfa using GBS markers and phenotypic selection for two years to indicate the potential of the GS method in enhancing genetic gain in alfalfa yield [33]. The GS for alfalfa yield conducted using different reference populations exhibited moderate prediction accuracy and the method was efficient [34].

Identifying significant marker(s) located near or at QTL for biomass and its introgression into elite germplasms for increasing the number of favorable alleles is valuable for biomass increase in alfalfa [2]. Furthermore, knowledge of correlations among various alfalfa traits and their genetic basis may allow simultaneous improvement of traits for high biomass and forage quality. Therefore, the objectives of this study were: (i) to identify QTLs controlling alfalfa TOF and spring yield (SY), (ii) to evaluate the phenotypic relationship between alfalfa crucial traits such as FD, WH, SY and TOF in a bi-parental population, and (iii) to compare the physical location of SNPs under detected QTLs to the *Medicago* reference genome for identifying the potential role of those loci in the phenotypic variation of the traits.

Materials and Methods

Mapping population development and phenotyping

Plant materials (F1, checks and parents), experimental design, and testing sites were described previously [7]. A total of 184 F1 progeny obtained by hand crossing two alfalfa cultivars (3010 ♀ x CW 1010 ♂) were confirmed for their parentage using five SSR markers. The plants were established at two environments Watkinsville (JPC farm) and Blairsville (BVL), GA using a RCBD design with three replications in August 2014. Four clones of each F1 genotypes and two parents prepared through stem cuttings were transplanted in a single plot. The check cultivars for FD and WH [35] were directly seeded in the field. Spring TOF was recorded every three days until the appearance of at least onset flower in all the four clones. Flowering data was taken for the spring of two years 2015 and 2017. In spring 2016, we did not record flowering data for Blairsville as we had to replant the new clones because the prior plants were impacted by freezing and herbicide application. Therefore, we analyzed data for only two years for both environments. The TOF was estimated in Julian calendar days to at least one flower in all four clones in a plot.

Wet and dry weights of spring first harvest biomass (SY) were collected in 2017 and 2018 using a swift machine forage harvester (Swift Machine and Welding Ltd., Saskatchewan, Canada). Additionally, three subsequent summer cuts after the first spring harvest were recorded in 2017 in both environments. The cumulative summer biomass (CSB) data from three summer cuts, after spring harvest, was analyzed. Because of frequent rainfall in Blairsville area, we could not harvest biomass in 2018 on time. The dry weight was estimated from bulked samples selected randomly from all three replications to observe to determine the moisture percentage at harvest. The phenotypic data for both TOF and yield was fitted to the generalized linear model to obtain least square (LS) means of traits [7]. Each dataset for the year and the environment was analyzed

separately because of genotype by environmental and year (G x E x Y) interactions. Correlation between various phenotypic traits were estimated using the Proc Corr procedure in SAS 9.4 [36]. Time of flowering recorded for two years (TOF015 and TOF017), SY recorded for two subsequent years (SY017 and SY018), FD recorded for two years (FD015 and FD017), seasonal dormancy assessed in a winter 2017 (WD017), and WH data for two years (WH016 and WH017) were analyzed and Pearson correlation coefficients (r) were determined (Table 3.2 and 3.3). The correlation between CSB and other traits was also evaluated for 2017. To validate the correlations obtained in the F1 population, the check cultivars were also compared to the same variables.

The effect of accumulated growing degree days (AGDD) on spring flowering time in alfalfa was assessed at base temperature of five degree Celsius. Previous report also used 41 °F (5 °C) as a base temperature for GDD calculation [37]. The GDD calculation for two seasons (2015 and 2017) and two environments beginning from January 1 of the year to the spring flowering date was summarized (Table 3.3). The AGDD was estimated using a formula by [38];

$$\text{AGDD} = \sum_{i=0}^{n} \left(\frac{\text{Tmax}-\text{Tmin}}{2}\right) - \text{Tb}$$

Where, Tmax, Tmin and Tb refer to maximum temperature (°C), minimum temperature (°C) and base temperature (5 °C), respectively. The base temperature (Tb) is a minimum threshold temperature where below which no growth occurs. Temperature data was obtained from the University of Georgia (UGA) weather station at Watkinsville-UGA, Oconee County, Georgia [39].

QTL mapping and putative homolog identification

The methods used for linkage map construction and QTL mapping were described previously [7]. The single dose allele (SDA) SNP markers that segregated in 1:1 (1/2 Aaaa:1/2 aaaa) and polymorphic to either one of the parents were separated from the set of raw SNP markers discovered

by GBS method. The parental linkage maps contained the SNPs polymorphic in CW 1010 parent and the maternal linkage maps were constructed with the SDA SNPs polymorphic to 3010 parent. The 32 linkage groups for each of the two parents were aligned with the *Medicago* reference genome (Mt4.0v2) using BLAST search. Four alfalfa homologs were grouped and randomly assigned for each *Medicago* chromosome. Then, the QTLs were mapped using LS means of TOF and SY as a phenotypic value. QTL mapping was conducted using the composite interval mapping (CIM) method in Windows QTL Cartographer version 2.5 [40].

Comparative analysis of genomic regions was performed using SNPs in the QTL peak and flanking regions. The SNPs associated with QTLs for both TOF and SY were subjected to BLAST search against *M. truncatula* genome (Mt4.0) (http://www.medicagohapmap.org/tools/blastform) and the best BLAST hit outputs were viewed on GBrowse [41]. The putative homologous genes for the QTL regions were identified and their relevant functions were searched in the literature (Table 3.7).

Results

Phenotypic assessment of TOF and yield

There were significant differences ($P < 0.05$) in TOF among F1 individuals in all environments and years (Table 3.1). The F1 population exhibited near normal distribution for each dataset. In 2015, the LS mean estimated for TOF in JPC population ranged from 112 to 130 days, whereas in 2017 it varied from 85 to 110 days (Table 3.1). In Blairsville, the LS mean for TOF varied from 146 to 163 days in 2015, and 118 to 136 days in 2017 (Table 3.1). The parent CW 1010 and 3010 did not show significant differences ($P \geq 0.05$) upon comparison of their TOF means by two-sample t-test (SAS 9.4). The dormant parent 3010 had slightly shorter average flowering days than the non-dormant parent CW 1010 (Table 3.1). However, we could not record TOF for CW 1010

from all replications in all seasons as we lost some of them due to heavy winter injury. Abundant transgressive segregants were present on both early and late flowering sides of the distribution. Further, significant G x E for TOF were observed for both 2015 and 2017 flowering data.

Variations in F1 individuals were observed for the spring yield (SY) and for three subsequent summer cuts (Table 3.1). The F1 progenies were significantly different ($P < 0.05$) for both SY and summer cuts as revealed by the analysis of variance (ANOVA). In the JPC location, the F1 LS mean estimated for SY in 2017 ranged from 0.33 to 1.9 kg/plant (Table 3.1). Similarly, in Blairsville the F1 individuals SY in 2017 ranged from 0.25 to 2.05 kg/plant. The LS means of SY for F1 exhibited near normal distribution for the datasets recorded for both environments. The dry weight estimated for randomly selected F1 plant samples, after oven drying at 60 °C for three days, showed nearly 30 % dry matter percentage in spring biomass harvest at both environment, which was decreased to 25 % for summer biomass.

Phenotypic correlations

A weak negative correlation was observed between TOF and FD in the alfalfa F1 population, indicating the genotypes with shorter days to flowering have possibly higher dormancy levels or non-dormant (Table 3.2 and 3.3). The correlation coefficient (r) calculated for different dormancy and flowering data is given in Table 3.2 and 3.3. In the JPC environment, the highest correlation coefficient ($r = -0.44$, $P < 0.01$) between TOF and dormancy was observed for datasets TOF017 and WD017. However, FD recorded in the fall (FD015 and FD016) showed a weaker relationship with TOF. Also, in Blairsville, the r calculated for TOF and FD exhibited a weak negative to non-significant ($P \geq 0.05$) correlations (Table 3.3). The highest r between FD and TOF for this environment was -0.13 ($P < 0.05$) (Table 3.3). We also analyzed the FD check cultivars for the TOF and FD relationship (data not given), but we could not observe any strong correlations between these

traits. Further, no significant difference ($P \geq 0.05$) was observed in TOF LS means estimated for dormant and non-dormant parents (Table 3.1). This suggests that spring TOF is not an index to predict FD in alfalfa. Nevertheless, the relationship may be specific to this mapping population, which in fact derived from well adapted dormant and non-dormant cultivars.

TOF in alfalfa F1 population also exhibited a weak negative correlation with the WH score assessed by visual rating in the field (Table 3.2 and 3.3). The negative correlation between TOF and WH indicates that the winter-hardy plants (lower WH score) showed maximum flowering later in the spring. The highest correlation value ($r = -0.43$, $P < 0.01$) was observed between TOF017 and WH017 (Table 3.2) for the JPC population. However, in the Blairsville environment the correlation between TOF and WH were mostly non-significant ($P \geq 0.05$). Although the correlation observed between TOF and WH was weak, it seemed that the winter-hardy plants may need longer time to reach maximum flowering stage.

Weak negative significant ($P < 0.05$) to non-significant ($P \geq 0.05$) correlations were observed between TOF and spring yield (SY) in both environments (Table 3.2 and 3.3). Similar relationship was also observed between TOF and CSB. Nevertheless, the correlation between TOF and SY (SY018) were non-significant ($P \geq 0.05$) (Table 3.2). Unlike other perennial grasses, alfalfa showed weaker negative correlation between TOF and spring biomass in F1 population.

Correlation between FD and SY

Dormancy assessed in the fall (FD015, FD016) and the winter (WD017) displayed significant positive correlation ($P < 0.05$) with SY (SY017 and SY018), suggesting that a fall non-dormant alfalfa genotype has higher yield even in spring (Table 3.2 and 3.3). In JPC environment, the significant correlation up to ($r = 0.29$, $P < 0.01$) was observed between WD017 and SY017, while in Blairsville the correlation coefficient up to ($r = 0.30$, $P < 0.01$) was obtained between

FD015 and SY017. The CSB obtained in three summer cuts did not display any significant correlations with FD (Table 3.2 and 3.3). However, CSB and SY showed a weak positive correlation in both environments and years.

Relationship between WH and SY

The direction of the phenotypic relationship between WH and SY altered according to the growing environment. In JPC, a weak positive correlation was observed between WH and SY, which means the winter-hardy plants (lower WH score) had a relatively low SY (Table 3.2). The correlation between WH and SY for JPC population ranged from 0.13 to 0.20 (Table 3.2). Nonetheless, in Blairsville, WH and SY exhibited a weak negative to non-significant ($P < 0.05$) correlations. This indicates that in Blairsville environment, the impact of winter damage is higher than in JPC, and the winter-hardy plants had relatively higher biomass in that environment.

TOF and GDD

Plants in Blairsville flowered approximately one month later than those at the JPC. The delayed flowering in BVL is likely due to freezing winter. In 2015, minimum average temperature at BVL was -12.6 °C compared to the -7 °C of JPC (Table 3.4). In 59 days of two winter months (Jan and Feb) in 2015, the BVL plants faced < 5 °C (Tb) for 45 days. The winter of 2017 was milder, which led plants to flower about four weeks earlier than 2015 at both sites (Table 3.4). Nevertheless, flowering time difference between plants at BVL and JPC was not changed. Therefore, if winter is severe, one should wait longer than usual for subsequent alfalfa spring regrowth harvest typically at early bloom stage.

The alfalfa population at BVL required higher AGDD than the population at JPC to reach flowering stage (Table 3.4) regardless of the severity of winter (Table 3.4). The higher AGDD requirement for BVL plants was most likely due to the longer chilling period in winters with several

days having below to near base temperature (Tb). Essentially, the temperatures that are not enough to break dormancy and start spring regrowth, but still higher than Tb contribute to cumulative GDD. Therefore, requirement of more AGDD and delayed flowering in BVL plants is more relevant to its extreme winter temperatures.

QTL Mapping

QTLs of spring TOF in alfalfa

Within 32 homologs of maternal parent 3010, we mapped 13 significant QTLs (LOD ≥ 3.0) for flowering time. The QTLs were coded as Tof-d1, Tof-d2, …, Tof-d13 to denote the QTLs of TOF detected for the 'dormant' parent. Because of nearly one-month difference in TOF between plants at JPC and BVL, we analyzed each environment and year data separately. The most important flowering QTL for 3010 parent, Tof-d7 (R^2 = 0.15), was for early flowering (- allele direction) and was detected on homolog 3B with a LOD value of 7.7 (Table 3.5). Out of the 13 flowering time QTLs detected for 3010 parent, eight QTLs showed positive effects on the phenotypic value (+ allele direction) and the remaining five QTLs had negative effects on phenotype or they are associated with earlier flowering (Table 3.5). In both environments, for 3010 parents, we detected QTLs for both (+ and -) phenotypic effect sides to control TOF. Most of the flowering QTLs were identified on homologs of chromosome seven, three and one, for 3010 parent (Table 3.5). A QTL was also identified on homolog 6D. There were two putative QTLs detected on homolog 7B with +ve allele direction (late flowering) for 3010 parent; however, they were not reported here because they were detected with slightly smaller LOD (LOD = 2.8) than the threshold (LOD ≥ 3.0).

For CW 1010 parent, total 12 QTLs of TOF were identified on different homologs of chromosome four, five, six, seven and eight (Table 3.5). The QTLs were coded as Tof-n1, Tof-n2, …, Tof-n12 to denote the QTLs of TOF for the 'non-dormant' parent. Out of the 12 QTLs, the

homologs of chromosome six (Figure 3.1) and seven harbored each four QTLs, two other QTLs were located on homologs 8B and remaining two QTLs were identified each on homolog 4B and 5B (Table 3.5). A QTL, Tof-n6, detected on homolog 7C for CW 1010 explained the highest percentage of phenotypic variance ($R^2 = 0.16$). Three flowering QTLs Tof-n5, Tof-n6 and Tof-n7 were identified as stable QTLs as they were detected in more than one environment and/or season. As in 3010, the flowering QTLs of CW 1010 carried both types of alleles (+ and -). Nine QTLs were identified for early flowering (- allele direction) and other three QTLs were identified for delayed flowering (+ allele direction) (Table 3.5). However, the QTLs for delayed flowering were detected only on homolog 6D for this parent. The peak and flanking markers of relevant QTLs are given in Table 3.5.

QTLs of SY and CSB in alfalfa

We detected 10 QTLs of SY within the homologs of 3010 parent, and named as SY-d1, SY-d2, …, SY-d10 (Table 3.6). All QTLs detected for 3010 parent had negative allele effects on the phenotype, suggesting that fall dormant progeny would have reduced biomass yield in the spring harvest. A QTL, SY-d1, detected with LOD = 5.8 and the $R^2 = 0.11$ was the most important QTL detected for SY in the 3010 parent, it had a negative effect on SY though. Also, SY associated QTLs were detected, however with negative effects, on homologs of chromosomes: 1, 3, 4 and 7 of 3010 parent (Table 3.6). On the genetic linkage maps of CW 1010, we mapped seven different QTLs (SY-n1, SY-n2, …., SY-n7) associated with SY (Table 3.6). The QTLs for CW 1010 spring yield were located on homologs 8A, 8B, 8C, 7A and 3D. All QTLs detected within the CW 1010 homologs possessed positive effects on SY, indicating that non fall-dormant parent also carries QTLs for higher spring yield. The markers in a QTL, SY-n5, explained the highest percentage of phenotypic variance ($R^2 = 0.13$) on SY for the CW 1010 parent.

Three QTLs (CSB-d1, CSB-d2, CSB-d3) associated with cumulative summer biomass (CSB) were mapped on the maternal linkage map. The QTLs were detected on homologs 3A, 4D and 8C, and each three QTLs detected for 3010 parent had a negative effect on phenotype. The QTL CSB-d1 explained the highest phenotypic variance ($R^2 = 0.10$) (Table 3.6). Similarly, three QTLs associated with CSB were also detected on homologs: 1C, 4D and 5B for CW 1010 parent. The QTLs CSB-n1 and CSB-n2 displayed positive effects on trait value, whereas a QTL CSB-n3 showed a negative effect. The QTL CSB-n1 of CW 1010 contributed the highest variance to the phenotype ($R^2 = 0.07$).

Identification of putative homologous genes

Several putative homologous genes and corresponding proteins were identified for SNP sequences of QTLs associated with TOF and SY using $BLAST_n$ search against Mt4.0, A17 reference genotype pseudomolecules database (Table 3.7) [41]. Putative homologs were declared if sequences were aligned with ≥ 95% identity as described previously [42]. We found 12 putative homologs associated with nine different flowering QTLs of 3010. Also, seven putative homologs associated with five different flowering QTLs for CW 1010 were identified (Table 3.7). Similarly, 10 homologs were identified for SNPs of eight different SY QTLs for 3010, and eight homologs were identified for six SY QTLs for CW 1010. For CSB, we found three homologs associated with two QTLs for 3010 and obtained six homologs of SNPs of three CSB QTLs for CW 1010. The putative homologous genes could be a target for crop improvement through MAS.

Discussion

F1 progeny segregation for TOF

Although mean flowering time of the two parents with contrasted dormancy were not significantly different ($P \geq 0.05$), segregation in the F1 mapping population was observed with a nearly normal distribution of their trait values. Segregated F1 individuals allowed us to map the trait.

Since the trait was mapped in an F1 population, we essentially mapped the variation within each of the parents, not between the two parents. Therefore, whether the two parents vary in the trait is not very relevant. Moreover, the QTLs for early and delayed flowering were detected in both parents, which most likely suggests the intermediate type of flowering time for both parents. Interestingly, the maternal parent 3010 flowered slightly earlier than the CW 1010 parent, but still the parent carried higher numbers of QTLs (8/13) for delayed flowering than early flowering (5/13). Nonetheless, very important flowering QTLs detected on 3010 genome were for early flowering, such as Tof-d7 (R^2 = 0.15) and Tof-d11 (R^2 = 0.09) (Table 3.5). Similarly, in CW 1010 genome, we found the early flowering QTLs, such as Tof-n6 (R^2 = 0.16) and Tof-n2 (R^2 = 0.14) as well as delayed flowering QTLs, such as Tof-n3 (R^2 = 0.09) and Tof-n4 (R^2 = 0.10). The non-dormant parent CW 1010 carried higher numbers of early flowering QTLs. Therefore, both parents could pass any kind of QTLs for TOF to their progeny. Nevertheless, early flowering is most likely present in non-dormant progeny compared to the dormant progeny because the chance of inheritance of early flowering QTLs from the non-dormant parent was higher. The weak phenotypic correlation, between non-dormancy and early flowering could be partly because of the same reason. Nevertheless, predicting FD based on TOF on alfalfa seems unrealistic, at least in this population, because of the lack of strong phenotypic correlation between the traits, and the presence of both early, and delayed flowering loci in both parents.

The presence of transgressive segregants on either side of flowering time could have different explanations. Since both parents carried QTLs for TOF and the QTLs were different, early flowering, intermediate and delayed flowering progeny were expected regardless of the variation in their parents. Assuming each parent carries a single QTL for TOF that delays flowering and the QTLs have similar effect, progeny that carry both QTLs could be very late flowering (additive), and

progeny that carry neither of the two QTLs could be early flowering. The parents, however, would have similar flowering times. Likewise, if a progeny carries an early flowering QTL from a parent and a delayed flowering QTL with similar effect from another parent, then that progeny could have intermediate TOF.

Correlation among agronomic and adaptation traits

Phenotypic correlations among agronomic and adaptation traits in alfalfa, such as SY, TOF, seasonal dormancy and WH reported in the study can be valuable for crop management and trait manipulation for crop improvement. For instance, developing a winter-hardy non-dormant alfalfa cultivar with early spring regrowth and flowering would be ideal to extend the growing period up to early winter, and to harvest fresh forage earlier in the spring. Previously, we described the possibility of simultaneous improvement of FD and WH [7]. This study revealed that the manipulation of flowering time, which means the harvest period, is likely in both dormant and non-dormant alfalfa. Furthermore, environment specific performance of the F1 population for their AGDD requirement, winter WH and SY correlation added useful information for future research.

Detection of evolutionarily conserved and novel TOF QTLs

We detected 25 flowering QTLs, 13 for 3010 parent and 12 for CW 1010 parent, and some of them were constantly expressed in multiple locations and/or years (Tof-n5, Tof-n6, and Tof-n7). Some flowering QTLs detected on both parental linkage maps were consistent with previously reported QTLs of flowering in *Medicago*. This indicates that these QTLs are evolutionarily conserved, and they enhance the validity of QTL detection. For instance, a flowering QTL Tof-n10 (19.3 – 31.5 cM) detected on 8B homolog relative genomic location with a *Medicago* QTL on chromosome 8 (12 – 23 cM) for LR4 population [9], and both induced early flowering. A flowering QTL Tof-d5 on sub-genome 7A for 3010 parent, which spanned 37.5 – 38.7 cM at 1-LOD support

interval (LSI) and 36.8 – 39.0 cM at 2-LSI detected in corresponding genomic region with a flowering QTL of *Medicago* on chromosome 7 reported previously in a recombinant inbred line population (LR1) [9]. These two flowering QTLs also exhibited similar function of inducing delayed flowering. Similarly, a stable alfalfa flowering QTL Tof-n7 (48.4 - 54.7 cM) on 7C for CW 1010 parent was identified in a relative genomic position of a *Medicago* flowering QTL (47-65 cM) on corresponding chromosome 7 detected previously in LR5 RIL population [9], both conferring early flowering. The QTL Tof-n6 also detected in corresponding genomic region with a flowering QTL of *Medicago* on chromosome 7 for LR1 population, but with opposite effect. Moreover, we identified other novel QTLs on different homologs of chromosome 7 for 3010 and CW 1010, such as Tof-d4, Tof-d8, Tof-d9, Tof-n8 and Tof-n9 (Table 3.5). Therefore, chromosome 7 and its homologs in alfalfa are very important genomic sites for flowering QTLs as in *M. truncatula* [13]. This chromosome 7 of *Medicago* also recognized for the presence of copies of flowering locus T (*FT*) in previous reports [9,13]. Nonetheless, several other novel QTLs of TOF detected in this study on sub-genomes of various alfalfa chromosomes added valuable genomic resources for MAS. Since we observed QTLs for both early flowering and delayed flowering, either trait can be improved. However, improving flowering time trait in alfalfa is purpose specific. Early flowering may be desirable for early spring cutting or delayed flowering may be a choice in the regions where early spring frost compromises plant regrowth.

Novel QTLs for SY and CSB

Total 17 SY associated alfalfa QTLs, ten for 3010 and seven for CW 1010 were detected in this study. There is limited information regarding alfalfa SY QTLs, and the QTLs mapped previously were mostly based on unsaturated and incomplete sets of genetic linkage maps. Robins et al. (2007) mapped alfalfa biomass production on genetic linkage maps constructed using restriction

fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers using single marker analysis [43]. They further mapped forage yield, plant height, and regrowth on the same genetic maps [44]. However, both mapping studies were carried on only eight alfalfa linkage groups, unlike the sub-genome level mapping in this study. Nevertheless, they detected markers associated with yield mainly on linkage groups 3, 4, 7 and 8, we also detected QTLs of SY in some homologs of these chromosomes for either parent (Table 3.6). The markers with positive effects on spring biomass, especially detected in the non-dormant parent, would be an essential target for enhancing alfalfa biomass. Further, the potential QTLs obtained for CSB such as CSB-n1 and CSB-n2, which have positive effects on biomass would be useful to enhance the cool season alfalfa yield in summer months.

Putative homologs as genomic resources

We identified 19 putative homologs associated with flowering, 18 homologs for SY and 9 homologs for CBS within genetic maps of 3010 and CW 1010 (Table 3.7). We also found a common *Medicago* homolog 'Medtr4g090600' for SNPs of a QTL of flowering (Tof-n11) as well as SY (SY-n3), which suggests the involvement of common pathways for TOF and SY in alfalfa. The number of *Medicago* putative homologous genes could be increased if we had scanned all SNPs under the QTL regions. This is because the genetic maps used in this study are dense and mostly consist of several SNPs under QTL regions. Relaxing stringency of LOD support interval (LSI), such as using 2-LSI instead of 1-LSI, could also enhance the numbers of SNPs associated with the traits. Functions of the putative homologs identified in this study on *Medicago* reference genome can also be searched in other model plant species. For instance, a putative homolog identified for TOF QTL Tof-d9 in this study, known as a bZIP transcription factor (Table 3.7), was previously reported as a candidate gene for flowering time in *Medicago* [9] and Arabidopsis [45]. Also, searching homologs

in updated databases of *Medicago* and alfalfa, such as *M. sativa* CADL v1.0, using the newer genomic tool box, for example, Alfalfa Breeder's Toolbox [46], would be helpful to enhance putative homologs search. Therefore, detailed study of putative homologs for their roles in biological pathways relevant to phenotypic variations is recommended. The genomic region with putative homologs could be focused for candidate gene search for the corresponding traits.

Conclusion

In this study, we mapped stable, novel and potential QTLs of important agronomic and adaptation traits on saturated genetic maps, which added valuable genomic resources for alfalfa improvement. The phenotypic correlations we reported for alfalfa traits under different environments would be valuable for crop management. As the QTLs identified in this study cover a set of GBS SNPs in their sub-genomes, homologous gene search can be expanded to other database of *Medicago* and alfalfa genomes available at the diploid level. The trait associated QTLs and SNPs can be used in accelerating alfalfa breeding to achieve higher genetic gain. Nonetheless, validation of the reported QTLs in sets of diverse genetic backgrounds and multiple environments is recommended.

Tables and Figures

Table 3.1. Range of LS means in F1 population for TOF, SY and CSB for three cuts. The LS means of both parents for respective traits are also given.

Trait	Environment	F1 (ψ)	3010 ($)	CW 1010 ($)
TOF015	JPC	112 - 130	117	119
TOF17	JPC	85 - 110	101	102
TOF015	BVL	146 - 163	149	151
TOF17	BVL	118 - 136	118	121
SY017	JPC	0.33 - 1.9	0.64	1.05
CSB	JPC	0.11 - 1.48	0.347	0.34
SY018	JPC	0.10 -1.35	0.83	0.86
SY017	BVL	0.25 - 2.05	1.08	0.7
CSB	BVL	0.05 - 0.75	0.37	0.25

ψ = Range of LS means of trait in F1 progeny; $ = Parental mean for traits under given environment and year

Table 3.2. Correlations between different phenotypic traits in a F1 mapping population at JPC, GA research site.

	TOF015	TOF017	SY017	CSB	SY018	FD015	FD016	WD017	WH016	WH017
TOF015		0.33**	-0.04^{NS}	-0.06^{NS}	-0.04^{NS}	-0.19**	-0.11*	-0.28**	-0.22**	-0.21**
TOF017			-0.17**	-0.12**	-0.02^{NS}	-0.16**	-0.28**	-0.44**	-0.33**	-0.43**
SY017				0.35**	0.36**	0.18**	0.18**	0.29**	0.13**	0.20**
CSB					0.18**	0.05^{NS}	0.0.01^{NS}	0.07^{NS}	0.06^{NS}	0.04^{NS}
SY018						0.24**	0.17**	0.26**	0.15*	0.14**

* P < 0.05, ** P < 0.01, ^{NS}non-signifiant

Table 3.3. Correlations between different phenotypic traits in an F1mapping population at Blairsville, GA research plot. The variables TOF, FD, WD, WH and yield were compared.

	TOF015	TOF017	SY017	CSB	FD015	FD016	WD017	WH016	WH017
TOF015		-0.05^{NS}	-0.03^{NS}	-0.03^{NS}	-0.010*	-0.11*	-0.05^{NS}	-0.11*	-0.02^{NS}
TOF017			-0.24**	-0.26**	-0.13*	-0.06^{NS}	-0.07^{NS}	-0.06^{NS}	-0.06^{NS}
SY017				0.37**	0.30**	0.15*	0.12*	-0.13*	-0.06^{NS}
CSB					0.11*	0.01^{NS}	-0.01^{NS}	-0.01^{NS}	-0.16**

* $P < 0.05$, ** $P < 0.01$, ^{NS} non-signifiant

Table 3.4. Alfalfa AGDD requirement to reach flowering in spring. This was estimated from the first day of the year, based on two locations (BVL and JPC). It seems that more AGDD is required for the BVL population than JPC is due to BVL harsh winter condition with frequent low-freezing temperature.

Environment	Year	Flowering Date	TOF (Days)	Days(ɬ)	T-max (¤)	T-min (ʊ)	Days < Tb (ψ)	AGDD (¥)
JPC	2015	13th April	112	84	12.5	- 7	28	641.1
JPC	2017	27th March	85	75	18.9	-2.5	6	587.6
BVL	2015	27th May	146	98	11.4	-12.6	45	876.1
BVL	2017	29th April	118	93	15.5	-8.8	18	729.6

ɬ = Effective days to flowering with temperature > Tb (5 °C); ¤ = Maximum average temperature (°C) in two winter months (Jan. and Feb.); ʊ = Minimum average temperature in (°C) two winter months (Jan. and Feb.); ¥ = AGDD from 1st January to the day of early flowering record, the AGDD was obtained by adding GDD of the effective days; and ψ = Total chilling days in two winter months with temperature < Tb.

Table 3.5. QTLs for TOF in alfalfa identified in an F1 (3010 x CW 1010) population using pseudo-testcross strategy and phenotypic data assessed in two years for TOF at two environments (JPC and BVL). The QTL code 'Tof-d' was given to flowering QTLs detected on linkage maps of dormant parent 3010, and the code 'Tof-n' was provided to flowering QTLs detected on linkage maps of the non-dormant parent CW 1010.

Parent	QTL code	Chr.	Location/Year	Peak Marker	Peak LOD	R^2	Allele Dir.	LSI (cM)	Flanking Markers
3010	Tof-d1	1D	JPC/ Π	MRG_14469273	4.4	0.08	-	34.9 - 36.9	TP56677-TP66486
3010	Tof-d2	1D	JPC/Π	TP60376	3.3	0.06	-	30.6 – 31.1	TP60376-TP41436
3010	Tof-d3	3C	JPC/Π	TP52465	3.5	0.06	+	38.1- 44.0	TP37583-TP72054
3010	Tof-d4	7A	JPC/Π	TP58371	3.3	0.06	+	27.3 - 30.4	TP58371-TP2134
3010	Tof-d5	7A	JPC/Π	TP24733	3.8	0.07	+	37.5 – 38.7	TP55743-TP34483
3010	Tof-d6	1A	JPC/β	TP85729	3.3	0.06	+	95.1 -96.6	TP5699-TP36877
3010	Tof-d7	3B	JPC/β	TP68861	7.7	0.15	-	42.5 -45.9	TP60221-TP68861
3010	Tof-d8	7A	JPC/β	TP28256	5.1	0.09	+	2.4 -6.1	TP28256-TP80202
3010	Tof-d9	7B	JPC/β	TP3421	4.8	0.09	+	23 -27.2	TP9376-TP3421
3010	Tof-d10	1A	BVL/Π	TP35274	3.3	0.07	+	88.9-91.3	TP52576-TP995
3010	Tof-d11	1B	BVL/Π	TP23433	3.8	0.09	-	45-49.7	TP23433-TP66714

3010	Tof-d12	3D	BVL/Π	TP18933	3.4	0.07	-	19.3-24.4	MRG_22559848-TP66479
3010	Tofd13	6D	BVL/β	MRG_2402742	3.7	0.08	+	16.2 – 23.0	TP16313-TP18699
CW 1010	Tof-n1	5B	JPC/Π	TP11856	4.1	0.07	-	75.3 – 79.4	TP80448-TP80460
CW 1010	Tof-n2	6B	JPC/Π	TP3310	7.1	0.14	-	17.2 – 21.2	TP3310-TP71145
CW 1010	Tof-n3	6D	JPC/Π	TP48161	4.8	0.09	+	54.8 – 56.4	MRG_5981048-TP6188
CW 1010	Tof-n4	6D	JPC/Π	TP49028	5.5	0.10	+	60.1 – 60.5	TP70280-TP66860
CW 1010	Tof-n5	6D	JPC/Π, BVL/ β	TP24444	3.8	0.08	+	65.9 – 66.8	TP64001- TP32647
CW 1010	Tof-n6	7C	JPC/Π, JPC/β	TP44666	8.0	0.16	-	42.9-44.7	TP45002- TP4972
CW 1010	Tof-n7	7C	JPC/Π, JPC/β	TP54614	4.1	0.07	-	48.4 – 54.7	TP38417- TP54614
CW 1010	Tof-n8	7B	JPC/β	TP9019	4.0	0.07	-	23.7-26.1	TP14107- MRG_9345022
CW 1010	Tof-n9	7B	JPC/β	TP36500	4.7	0.09	-	30.8-31.6	TP36500-MRG_25777286
CW 1010	Tof-n10	8B	BVL/Π	TP76596	3.6	0.08	-	19.3 – 31.5	TP76596-TP75547
CW 1010	Tof-n11	8B	BVL/Π	TP75547	3.1	0.07	-	31.5 – 38.9	TP75547-TP25170
CW 1010	Tof-n12	4B	BVL/ β	TP66329	4.6	0.09	-	5.5-12.8	TP88701-TP57672

Π = Year 2015; β = Year 2017; Chr. = Chromosome; Dir. = Direction; LSI = 1-LOD support interval in cM unit

Table 3.6. QTLs for SY and CBS in alfalfa mapped on 3010 and CW 1010 genetic linkage maps using pseudo-testcross strategy

Parent	QTL code	Chr.	Location/Year	Peak Marker	Peak LOD	R^2	Allele Dir.	LSI (cM)	Flanking Markers
3010	SY-d1	3A	JPC/β	TP2592	5.8	0.11	-	56.5-57.1	TP2592-TP37840
3010	SY-d2	3A	JPC/β	TP83334	4.1	0.08	-	63.5-64.9	TP59541-TP83334
3010	SY-d3	4D	JPC/β	MRG_4464574	5.1	0.10	-	67.1-70.5	MRG_4464482-TP43038
3010	SY-d4	7A	JPC/β	TP51377	4.9	0.10	-	76.2-83.2	TP51377- TP47813
3010	SY-d5	7A	JPC/ϕ	MRG_10667023	4.1	0.08	-	40.9-43.2	TP30610- MRG_10666968
3010	SY-d6	7C	JPC/ϕ	TP66942	3.1	0.06	-	88.8-91.4	TP87634-TP66942
3010	SY-d7	7D	JPC/ϕ	TP34947	4.5	0.09	-	33.7-35.8	TP14368-TP40888
3010	SY-d8	1A	BVL/β	TP46942	3.7	0.07	-	72-72.9	TP72089-TP46942
3010	SY-d9	1B	BVL/β	MRG_25771949	3.2	0.06	-	76.7-79.7	TP6511-TP34670
3010	SY-d10	1D	BVL/β	TP1567	3.7	0.07	-	26.4-26.9	TP89308-TP1567
3010	CSB-d1	3A	JPC/β	TP16385	4.2	0.10	-	0-5.5	TP16385-TP32175
3010	CSB-d2	4D	JPC/β	TP32956	3.1	0.06	-	103.5–106.8	TP83938-TP55849
3010	CSB-d3	8C	JPC/β	TP27142	3.5	0.07	-	43.7-46.3	TP66239-TP27142
CW 1010	SY-n1	3D	JPC/β	TP85451	3.4	0.10	+	5.3-7.6	TP11255-TP85451
CW 1010	SY-n2	8A	JPC/β	TP45400	3.0	0.06	+	24.7-25.8	TP9008-TP41903

CW 1010	SY-n3	8B	JPC/β	TP25170	3.0	0.06	+	34.5-44.8	TP25170-TP86491
CW 1010	SY-n4	8C	JPC/ϕ	TP27703	4.9	0.12	+	33.3-35.9	TP31047-TP27703
CW 1010	SY-n5	8C	JPC/ϕ	TP77807	4.0	0.13	+	51.4-52.5	TP40142-TP77807
CW 1010	SY-n6	7A	BVL/β	MRG_28464923	4.0	0.07	+	32.4-34	TP57427-MRG_12020287
CW 1010	SY-n7	7A	BVL/β	TP50516	4.7	0.08	+	40.1-44.2	TP13897-MRG_4633212
CW 1010	CSB-n1	1C	JPC/β	TP10914	3.5	0.07	+	13.4-17.4	TP11572-TP42278
CW 1010	CSB-n2	4D	JPC/β	TP83595	3.3	0.06	+	23.2-26.1	TP83595-TP70955
CW 1010	CSB-n3	5B	JPC/β	TP26255	3.2	0.06	-	34.9-37.5	TP26255-TP18857

β = Year 2017; ϕ = Year 2018

Table 3.7. Putative homologs identified using sequences of SNPs under peak and flanking regions of QTLs for TOF, SY and CSB in both parents.

Parent	QTL code	Markers	Putative Homologs and Functions	% Identity	E-value	*M. truncatula* Homologs
3010	Tof-d1	TP66486	Myosin motor domain protein and Dil domain protein	98.36	4e-23	Medtr1g070400
3010	Tof-d2	TP60376	P-loop nucleoside triphosphate hydrolase superfamily protein	98.44	9e-25	Medtr1g075200
3010	Tof-d5	TP34483	Pre-mRNA splicing factor-like protein	100.00	2e-26	Medtr7g068630
3010	Tof-d6	TP36877	Chitinase	100.00	2e-26	Medtr1g099320
3010	Tof-d7	TP60221	Hypothetical protein	96.88	4e-23	Medtr8g064300
3010	Tof-d8	TP28256	U6 snRNA-associated-like-Smprotein	100.00	2e-26	Medtr8g058537
3010	Tof-d8	TP80202	Translational activator GCN1-like protein	100.00	2e-26	Medtr7g116425
3010	Tof-d9	TP3421	bZIP transcription factor	100.00	2e-26	Medtr7g088090
3010	Tof-d9	TP9376	Pre-mRNA-splicing factor SLU7-like protein	100.00	2e-26	Medtr7g096940
3010	Tof-d10	TP35274	Carbohydrate-binding X8 domain protein	96.88	4e-23	Medtr1g084820
3010	Tof-d10	TP995	Importin-like protein	96.43	1e-18	Medtr7g021500
3010	Tof-d11	TP23433	Exocyst complex component sec15B	98.44	9e-25	Medtr1g050505
3010	SY-d1	TP2592	LOB domain protein	96.88	4e-23	Medtr3g452660

3010	SY-d2	TP83334	Nudix hydrolase-like protein	98.44	9e-25	Medtr3g437740
3010	SY-d4	TP51377	RS2-interacting KH protein, putative	100.00	2e-26	Medtr7g013700
3010	SY-d4	TP47813	Peptide/nitrate transporter	98.44	9e-25	Medtr7g010820
3010	SY-d6	TP66942	Transcription factor	95.31	7e-21	Medtr7g092510
3010	SY-d7	TP14368	RING/U-box protein	96.88	4e-23	Medtr7g056183
3010	SY-d8	TP46942	Succinyl-CoA ligase [ADP-forming] subunit beta	100.00	2e-26	Medtr1g069645
3010	SY-d9	TP6511	Plastid transcriptionally active protein	100.00	2e-26	Medtr1g079525
3010	SY-d9	TP34670	Alpha/beta hydrolase family protein	96.72	2e-21	Medtr1g088470
3010	SY-d10	TP89308	Lon protease S16 carboxy-terminal proteolytic domain protein	95.24	7e-21	Medtr1g083990
3010	CSB-d2	TP32956	1-aminocyclopropane-1-carboxylate oxidase-like protein	100.00	2e-26	Medtr4g099390
3010	CSB-d2	TP83938	Phosphatase 2C family protein	98.39	1e-23	Medtr4g118340
3010	CSB-d3	TP27142	C2H2-type zinc finger protein, putative	96.88	4e-23	Medtr4g057230
CW 1010	Tof-n5	TP24444	Cytochrome P450 family protein	96.88	4e-23	Medtr1g116890
CW 1010	Tof-n5	TP64001	Granule bound starch synthase	98.39	1e-23	Medtr6g012380
CW 1010	Tof-n5	TP32647	Pentatricopeptide (PPR) repeat protein	95.31	2e-21	Medtr6g022140
CW 1010	Tof-n6	TP45002	Group 1 family glycosyltransferase	100.00	2e-26	Medtr7g067340
CW 1010	Tof-n7	TP54614	Pre-mRNA splicing factor-like protein	98.44	9e-25	Medtr7g068630
CW 1010	Tof-n8	TP14107	Det1 complexing ubiquitin ligase	95.31	2e-21	Medtr7g091260
CW 1010	Tof-n11	TP25170	Polyol/monosaccharide transporter 1	100.00	2e-26	Medtr4g090600*

CW 1010	SY-n1	TP11255	CCCH-type zinc finger protein, putative	98.44	9e-25	Medtr3g464260
CW 1010	SY-n2	TP41903	Armadillo repeat only protein	100.00	2e-26	Medtr4g073830
CW 1010	SY-n3	TP25170	Polyol/monosaccharide transporter 1	100.00	2e-26	Medtr4g090600*
CW 1010	SY-n3	TP86491	Pentatricopeptide (PPR) repeat protein	100.00	2e-26	Medtr8g106950
CW 1010	SY-n4	TP27703	ATP-dependent helicase BRM	100.00	2e-26	Medtr8g030550
CW 1010	SY-n5	TP77807	Trafficking protein particle complex subunit-like protein	100.00	2e-26	Medtr8g027700
CW 1010	SY-n7	TP50516	Pyruvate decarboxylase	95.31	7e-21	Medtr7g069500
CW 1010	SY-n7	TP13897	ARM repeat protein	96.88	4e-23	Medtr7g075940
CW 1010	CSB-n1	TP10914	XS domain protein	98.44	9e-25	Medtr1g492940
CW 1010	CSB-n1	TP11572	Octicosapeptide/phox/Bem1p family protein	98.44	9e-25	Medtr1g109470
CW 1010	CSB-n1	TP42278	Hypothetical protein	96.88	4e-23	Medtr1g079830
CW 1010	CSB-n2	TP70955	60 kDa inner membrane protein	98.44	9e-25	Medtr4g107330
CW 1010	CSB-n3	TP26255	Endoribonuclease E-like protein	96.88	4e-23	Medtr5g030900
CW 1010	CSB-n3	TP18857	Homeobox domain protein	96.88	4e-23	Medtr2g014490

* *M. truncatula* homolog identified for corresponding SNP sequence of two QTLs (Tof-n11 and SY-n3). The Tof-n11 is a flowering QTL and SY-n3 is a QTL for summer yield in the same CW 1010 parent

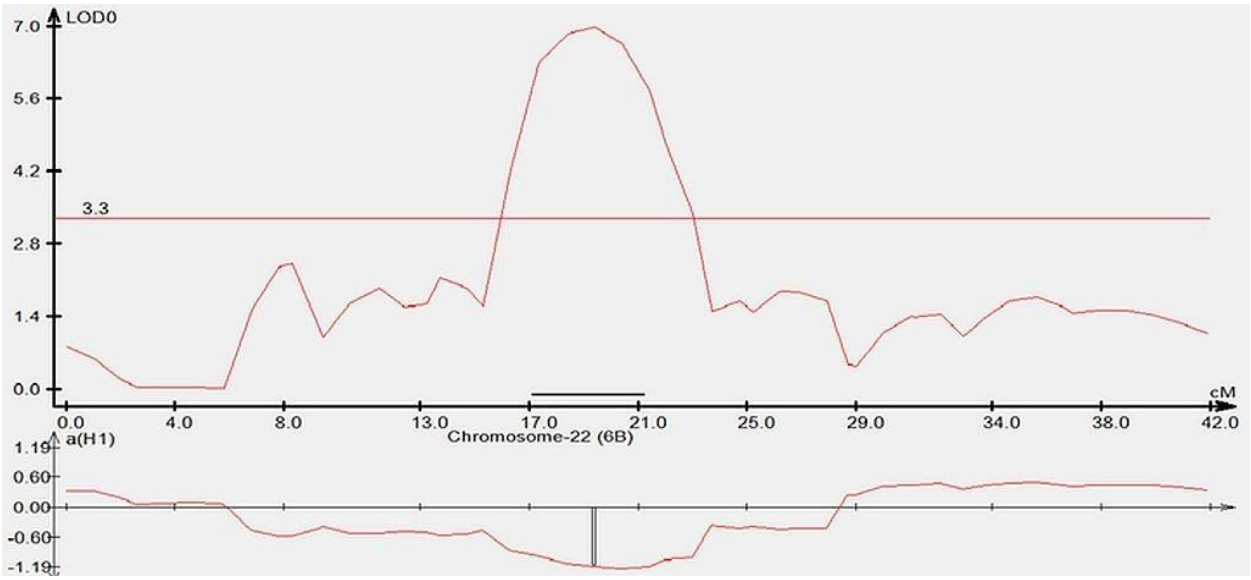


Figure 3.1. QTL peak for the flowering QTL Tof-n2 detected on chromosome 6B at LOD = 7.1 for the parent CW 1010. Since the Tof-n2 has an effect in negative direction, the QTL induces early flowering (shorter days to flowering).

References

1. **Li X, Wei Y, Acharya A, Jiang Q, Kang J, Brummer EC** (2014) A saturated genetic linkage map of autotetraploid alfalfa (Medicago sativa L.) developed using genotyping-by-sequencing is highly syntenous with the Medicago truncatula genome. G3 (Bethesda, Md) 4 (10):1971-1979. doi:10.1534/g3.114.012245

2. **Ray IM, Monteros MJ, Julier B, Sledge MK, Brummer EC** (2018) Identification of Consensus Regions Associated with Shoot Biomass Production in the Medicago Genome. Crop Science 58 (3):1037-1060. doi:10.2135/cropsci2017.11.0657

3. **Rock KP, Thelemann RT, Jung H-JG, Tschirner UW, Sheaffer CC, Johnson GA** (2009) Variation due to Growth Environment in Alfalfa Yield, Cellulosic Ethanol Traits, and Paper Pulp Characteristics. BioEnergy Research 2 (3):79-89. doi:10.1007/s12155-009-9035-0

4. https://alfalfa.ucdavis.edu/+symposium/2017/PDFfiles/Russelle%20Michael.pdf

5. **Adhikari L, Razar RM, Paudel D, Ding R, Missaoui AM** (2017) Insights into Seasonal Dormancy of Perennial Herbaceous Forages. American Journal of Plant Sciences Vol.08No.11:31. doi:10.4236/ajps.2017.811179

6. **Brummer EC, Shah MM, Luth D** (2000) Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. Crop Science 40 (4):971-977

7. **Adhikari L, Lindstrom OM, Markham J, Missaoui AM** (2018) Dissecting Key Adaptation Traits in the Polyploid Perennial Medicago sativa Using GBS-SNP Mapping. Frontiers in Plant Science 9 (934). doi:10.3389/fpls.2018.00934

8. **Li X, Alarcón-Zúñiga B, Kang J, Tahir N, Hammad M, Jiang Q, Wei Y, Reyno R, Robins JG, Brummer EC** (2015) Mapping fall dormancy and winter injury in tetraploid alfalfa. Crop Science 55 (5):1995-2011

9. **Pierre J-B, Huguet T, Barre P, Huyghe C, Julier B** (2008) Detection of QTLs for flowering date in three mapping populations of the model legume species Medicago truncatula. Theoretical and Applied Genetics 117 (4):609-620. doi:10.1007/s00122-008-0805-4

10. http://extension.uga.edu/publications/detail.html?number=B1350&title=Alfalfa% 20Management% 20in%20Georgia

11. https://crops.extension.iastate.edu/cropnews/2010/05/when-make-first-spring-cut-alfalfa-and-mixed-alfalfagrass

12. **Ridge S, Deokar A, Lee R, Daba K, Macknight RC, Weller JL, Tar'an B** (2017) The Chickpea Early Flowering 1 (Efl1) Locus Is an Ortholog of Arabidopsis ELF3. Plant Physiology 175 (2):802-815. doi:10.1104/pp.17.00082

13. **Weller JL, Ortega R** (2015) Genetic control of flowering time in legumes. Frontiers in Plant Science 6:207. doi:10.3389/fpls.2015.00207

14. https://www.alfalfa.org/pdf/howanalfalfaplantdevelops.pdf

15. **Grillo MA, Li C, Hammond M, Wang L, Schemske DW** (2013) Genetic architecture of flowering time differentiation between locally adapted populations of Arabidopsis thaliana. New Phytologist 197 (4):1321-1331. doi:doi:10.1111/nph.12109

16. **Ivanicova Z, Jakobson I, Reis D, Safar J, Milec Z, Abrouk M, Dolezel J, Jarve K, Valarik M** (2016) Characterization of new allele influencing flowering time in bread wheat introgressed from Triticum militinae. New biotechnology 33 (5 Pt B):718-727. doi:10.1016/j.nbt.2016.01.008

17. **Hori K, Matsubara K, Yano M** (2016) Genetic control of flowering time in rice: integration of Mendelian genetics and genomics. Theor Appl Genet 129 (12):2241-2252. doi:10.1007/s00122-016-2773-4

18. **Chardon F, Virlon B, Moreau L, Falque M, Joets J, Decousset L, Murigneux A, Charcosset A** (2004) Genetic Architecture of Flowering Time in Maize As Inferred From Quantitative Trait Loci Meta-analysis and Synteny Conservation With the Rice Genome. Genetics 168 (4):2169-2185. doi:10.1534/genetics.104.032375

19. **Reeves PH, Coupland G** (2001) Analysis of Flowering Time Control in Arabidopsis by Comparison of Double and Triple Mutants. Plant Physiology 126 (3):1085-1091. doi:10.1104/pp.126.3.1085

20. **Laurie RE, Diwadkar P, Jaudal M, Zhang L, Hecht V, Wen J, Tadege M, Mysore KS, Putterill J, Weller J** (2011) The Medicago truncatula FLOWERING LOCUS T homologue, MtFTa1, is a key regulator of flowering time. Plant Physiology:pp. 111.180182

21. **Zhang T, Chao Y, Kang J, Ding W, Yang Q** (2013) Molecular cloning and characterization of a gene regulating flowering time from Alfalfa (Medicago sativa L.). Molecular Biology Reports 40 (7):4597-4603. doi:10.1007/s11033-013-2552-0

22. **Herrmann D, Barre P, Santoni S, Julier B** (2010) Association of a CONSTANS-LIKE gene to flowering and height in autotetraploid alfalfa. Theoretical and Applied Genetics 121 (5):865-876. doi:10.1007/s00122-010-1356-z

23. **Gao R, Gruber MY, Amyot L, Hannoufa A** (2018) SPL13 regulates shoot branching and flowering time in Medicago sativa. Plant Molecular Biology 96 (1):119-133. doi:10.1007/s11103-017-0683-8

24. **Chao Y, Zhang T, Yang Q, Kang J, Sun Y, Gruber MY, Qin Z** (2014) Expression of the alfalfa CCCH-type zinc finger protein gene MsZFN delays flowering time in transgenic Arabidopsis thaliana. Plant Science 215-216:92-99.doi.org/10.1016/j.plantsci.2013.10.012

25. **Chao Y, Yang Q, Kang J, Zhang T, Sun Y** (2013) Expression of the alfalfa FRIGIDA-Like Gene, MsFRI-L delays flowering time in transgenic Arabidopsis thaliana. Molecular Biology Reports 40 (3):2083-2090. doi:10.1007/s11033-012-2266-8

26. **Tarantino LM, Eisener-Dorman AF** (2011) Forward genetic approaches to understanding complex behaviors. Behavioral Neurogenetics. Springer, pp 25-58

27. **Cunningham SM, Volenec JJ, Teuber LR** (1998) Plant Survival and Root and Bud Composition of Alfalfa Populations Selected for Contrasting Fall Dormancy. Crop Science 38 (4):962-969. doi:10.2135/cropsci1998.0011183X003800040014x

28. **Stout DG, Hall JW** (1989) Fall growth and winter survival of alfalfa in interior british columbia. Canadian Journal of Plant Science 69 (2):491-499. doi:10.4141/cjps89-060

29. **McCord P, Gordon V, Saha G, Hellinga J, Vandemark G, Larsen R, Smith M, Miller D** (2014) Detection of QTL for forage yield, lodging resistance and spring vigor traits in alfalfa (Medicago sativa L.). Euphytica 200 (2):269-279. doi:10.1007/s10681-014-1160-y

30. **Ray IM, Han Y, E L, Meenach CD, Santantonio N, Sledge MK, Pierce CA, Sterling TM, Kersey RK, Bhandari HS, Monteros MJ** (2015) Identification of Quantitative Trait Loci for Alfalfa Forage Biomass Productivity during Drought Stress. Crop Science 55 (5):2012-2033. doi:10.2135/cropsci2014.12.0840

31. **Li X, Wei Y, Moore KJ, Michaud R, Viands DR, Hansen JL, Acharya A, Brummer EC** (2011) Association Mapping of Biomass Yield and Stem Composition in a Tetraploid Alfalfa Breeding Population. The Plant Genome 4 (1):24-35. doi:10.3835/plantgenome2010.09.0022

32. **Sakiroglu M, Brummer EC** (2017) Identification of loci controlling forage yield and nutritive value in diploid alfalfa using GBS-GWAS. Theoretical and Applied Genetics 130 (2):261-268. doi:10.1007/s00122-016-2782-3

33. **Li X, Wei Y, Acharya A, Hansen JL, Crawford JL, Viands DR, Michaud R, Claessens A, Brummer EC** (2015) Genomic Prediction of Biomass Yield in Two Selection Cycles of a Tetraploid Alfalfa Breeding Population. The Plant Genome 8 (2). doi:10.3835/plantgenome2014.12.0090

34. **Annicchiarico P, Nazzicari N, Li X, Wei Y, Pecetti L, Brummer EC** (2015) Accuracy of genomic selection for alfalfa biomass yield in different reference populations. BMC Genomics 16:1020. doi:10.1186/s12864-015-2212-y

35. https://www.alfalfa.org/pdf/2014%20NAFA%20Variety%20Leaflet.pdf

36. SAS Institute Inc 2013. Cary, NC: SAS Institute Inc.

37. **Sanderson MA, Karnezos TP, Matches AG** (1994) Morphological Development of Alfalfa as a function of Growing Degree Days. Journal of Production Agriculture 7 (2):239-242. doi:10.2134/jpa1994.0239

38. **Iwata F** (1984) Heat unit cocept of maturity. In: Physilogical Aspects of Dry Land Farming. Gupta VS (Ed) Pp 351-370, Oxford and IBH

39. http://weather.uga.edu/index.php?content=calculator&variable=CC&site=WATUGA

40. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

41. http://www.medicagohapmap.org/fgb2/gbrowse/mt40/?

42. **Ray I, Monteros M, Julier B, Sledge M, Brummer E** (2018) Identification of Consensus Regions Associated with Shoot Biomass Production in the Medicago Genome. Crop Science

43. **Robins JG, Luth D, Campbell TA, Bauchan GR, He C, Viands DR, Hansen JL, Brummer EC** (2007) Genetic mapping of biomass production in tetraploid alfalfa. Crop science 47 (1):1-10

44. **Robins JG, Bauchan GR, Brummer EC** (2007) Genetic mapping forage yield, plant height, and regrowth at multiple harvests in tetraploid alfalfa (Medicago sativa L.). Crop science 47 (1):11-18

45. **Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T** (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science 309 (5737):1052-1056. doi:10.1126/science.1115983

46. https://www.alfalfatoolbox.org/

CHAPTER 4

QUANTITATIVE TRAIT LOCI MAPPING OF LEAF RUST RESISTANCE IN TETRAPLOID ALFALFA

Adhikari L, Missaoui AM (2018). Submitted to *Physiological and Molecular Plant Pathology*.

Highlights

- Eight QTLs associated with alfalfa rust resistance were detected using GBS of a pseudo-testcross mapping population.
- In this population, the inheritance of rust resistance caused by *Uromyces striatus* was polygenic and incomplete.
- The sequences of single nucleotide polymorphisms (SNPs) in the QTL regions exhibited homology with some genes that were associated with plant defense mechanism against fungal diseases in *M. truncatula*.
- In this study, phenotypic and genotypic correlations of rust severity with seasonal dormancy and winter hardiness were observed. However, the relation could be specific to the F1 population under consideration.

Abstract

Alfalfa leaf rust caused by the fungal pathogen *Uromyces striatus* results in substantial yield loss when harvesting is delayed. In Georgia, alfalfa leaf rust has been reported for the first time in 1960's. However, no additional research has been documented on pathogenesis and resistance mechanisms present in the host. In November 2016, we noticed a massive rust outbreak in an alfalfa population with an extensive coverage of rust spores on plants. The parents showed different reactions to rust infection as well as the progeny. F1 mapping population, designed to study fall dormancy (FD) and winter hardiness (WH) study, allowed us to investigate the genetic basis of leaf rust resistance in alfalfa. The objective of this work is to map quantitative trait loci (QTL) associated with alfalfa leaf rust resistance using genotyping-by-sequencing (GBS) SNP markers in a biparental F1 mapping population. The population was developed by crossing alfalfa cultivars, 3010 (♀) and CW 1010 (♂). The female parent 3010

displaying high susceptibility to rust while the male parent CW 1010 was moderately resistant. The mapping population consisted of 184 F1 that were cloned into 3 replications of 4 plants in each row and planted at Watkinsville and Blairsville, GA in a randomized complete block design (RCBD). Phenotyping rust severity (RS) was based on the NAAIC visual rating scores of 1 to 5. GBS library were prepared with a single digestion of DNA using *ApeKI* enzyme and SNP discovery was based on alignment with *M. truncatula* genome sequence. As the two parents represent are contrasting for FD and WH, we also estimated the correlations of RS with FD and WH scores. A weaker negative correlation (r = -0.26) between FD level and RS score were observed. Similarly, RS showed a weak negative correlation (r = -0.27) with WH. The dormant and winter-hardy plants appeared more susceptible to alfalfa leaf rust than the dormant and cold sensitive parent. Five alfalfa rust QTLs were identified in the CW 1010 genetic map and three QTLs were identified in the 3010 parent. The most important QTL Us-RustR1 explained 13% (R^2 = 0.13) of the variance. Altogether, the five QTLs detected for CW 1010 explained 38% of total phenotypic variation. While the three QTLs detected for the susceptible 3010 parent explained 21% of total phenotypic variation with the highest R^2 = 0.08 for Us-RustS1 on homolog 1A. This study clearly suggests the polygenic inheritance and incomplete rust resistance in alfalfa. Comparative genome analysis of QTL regions using sequences of causal SNPs revealed homology to *M. truncatula* sequences that were previously reported for their role in plant defense mechanism in other plants. Validation of the QTLs in larger population for multi-year data as well as fine mapping of the QTL regions could be the next step of this research. The markers presented in this study would be valuable resources for alfalfa genetic improvement via marker-assisted selection (MAS).

Keywords: Alfalfa, GBS, Rust, *Uromyces*, QTL Mapping, SNPs

Abbreviations

BLAST = Basic local alignment search tool
FD = Fall dormancy
FDR = Fall dormancy rating
GBS = Genotyping-by-sequencing
LS = Least square
LSI = LOD support interval
MAS = Marker-assisted selection
PKG = Phosphoglycerate kinase
QTL = Quantitative trait loci
RS = Rust severity
SDA = Single dose allele
SRA = Sequence read archive
WH = Winter hardiness

Introduction

Alfalfa (*Medicago sativa* L.) is a perennial, allogamous, and autotetraploid (2n = 4x = 32) legume grown for forage and animal feed [1,2]. Leaf rust caused by *Uromyces striatus*, a fungus species of the *Pucciniaceae* family, impedes alfalfa growth and development leading to substantial yield loss [3]. Leaves, petioles and sometimes stems of rust infected alfalfa display masses of reddish brown spores from circular pustules [4,5], and the spores can be scrubbed and collected easily from infected alfalfa. The spores overwinter and when the environment becomes favorable, they spread to alfalfa fields causing disease outbreaks [4,6]. Leaf shriveling, premature defoliation, biomass quality deterioration, and yield reduction are visible impacts of severe rust in alfalfa. Rust also reduces the persistence of established alfalfa and the disease pressure on newly planted seedlings results in plant death and stand thinning. Alfalfa leaf rust was reported in Georgia for the first time in 1960's [7,8]. However, limited information is available on the rust impact on forage and seed yield of alfalfa in Georgia and the southeastern

US. Because of the hot and humid summer and fall, the Georgia weather seems favorable for the alfalfa rust pathogen.

Environmental factors such as temperature, leaf wetness, humidity and light are crucial conditions for rust infection and outbreak in alfalfa. Temperature and leaf wetness affect initial pathogen invasion efficiency, their latent period, and rates of pustule appearance on alfalfa leaves [3]. Aggressive growth of *U. striatus* was observed at temperatures ranging from 21 to 29 °C and high humidity conditions [9]. Together with growing environment, the rate of rust infection on alfalfa may also fluctuate with the host cellular properties. Moderate to a weak relationship was reported between acid detergent lignin (ADL) content in alfalfa leaves and rust resistance [10]. Resistance to *U. striatus* resistance in *M. truncatula* (here and after called only *Medicago*) was believed to be due to the ability of some accessions to restrict haustorium formation by aborting fungal colonies in earlier stages [11] Alfalfa rust can be controlled by agronomic practices or using resistant cultivars. Controlling *U. striatus* alternate hosts such as cypress spurge (*Euphorbia cyparissias*) and leafy spurge (*Euphorbia esula*)) was suggested to reduce the pathogen load in the field [12,4]. The application of commercial fungicides and timely clipping are other useful ways to effectively control alfalfa rust. Moreover, applying glyphosate was suggested as another method for controlling *U. striatus* in glyphosate resistant alfalfa[13]. Nevertheless, planting rust resistant cultivars is the most sustainable way to protect alfalfa against rust.

With the advent of next-generation sequencing and increasing options of genomic tools and resources, marker-assisted selection (MAS) has become a popular approach for developing plant cultivars with desired characteristics. Detecting causal alleles or quantitative trait loci (QTL) and associated molecular markers for rust resistance requires a standard set of phenotypic

data with sufficient markers. Because alfalfa is an autotetraploid, mapping QTL in F1 progenies using single dose allele (SDA) markers with the pseudo-testcross mapping strategy is common [14]. Identifying genes or QTLs underlying rust resistance and dissection of the genetic loci to understand the resistance mechanism have been done in a wide range of crops, including wheat (*Triticum aestivum* L.) [15], soybean (*Glycine max* L.) [16], barley (*Hordeum vulgare* L.) [17], maize (*Zea mays* L.), and sorghum (*Sorghum bicolor* L.) [18]. Rust resistance mechanisms in *Medicago*, a closer alfalfa relative, has also been reported [19,20]. The synteny between alfalfa and *Medicago* genes facilitates finding physical locations and homology information of alfalfa rust related SNPs in the *Medicago* genome. Moreover, *U. striatus* infects both *Medicago* and alfalfa, therefore similar genetic and genomic mechanisms may be underlying rust resistance in both species. Nevertheless, there is no information available on the genetic and genomic basis of rust resistance in alfalfa. Therefore, the objectives of this study are; i) to map QTL associated with alfalfa leaf rust resistance using genotyping-by-sequencing (GBS) SNPs; and ii) to identify putative homologs of the causal SNPs and QTLs sequences on the *Medicago* reference genome via comparative genome analysis.

Materials and Methods

In late fall 2016, we had an outbreak of leaf rust in an alfalfa field where an F1 alfalfa mapping population was planted in Watkinsville, GA [14]. The infection quickly spread over the entire field (Figure 4.1), which allowed for the ability to score the lesions on every progeny of the population. The rust invasion occurred when the higher temperature was slightly above 26.5 °C and the lower temperature was about 14 °C with no rain and low humidity [21]. Details of the F1 mapping population used in this study were described previously [14]. Briefly, an F1 population of 184 was progenies was developed by crossing two alfalfa cultivars, 3010 and CW

1010. The progeny plants were named as suffix “ALF” followed by their plant number, such as, ALF104. The female parent 3010 is dormant and winter-hardy, while the male parent CW 1010 is non-dormant and winter susceptible. The true hybrid status of each F1 plant was confirmed using SSR markers. The parents and progenies were grown at two locations, Watkinsville and Blairsville, Georgia using a RCBD design with 3 replications.

Rust severity (RS) rating was assigned based on visible signs of disease presence and intensity (Table 4.1), which was a slight modification of the North American Alfalfa Improvement Conference (NAAIC) protocol [22]. To improve the accuracy of the rating, the mapping population was scored twice with different people for two consecutive days. The average rust scores were estimated from the two datasets. Magnified images of rust infected leaves from each parent were taken using a microscope to aid in the scoring. Phenotypic data of rust scores was analyzed using SAS 9.4 (SAS Institute, 2004). The least square (LS) means for all genotypes was estimated using PROC GLM [14]. The LS means were used as the trait value in QTL analysis.

DNA extraction, GBS library preparation, sequencing and raw sequence data processing as well as single nucleotide polymorphisms (SNP) marker discovery and genetic map construction were described previously [14]. The DNA raw reads from Next Seq (150 Cycles) PE75 High Output flow cell were deposited to NCBI SRA (https://www.ncbi.nlm.nih.gov/sra/SRP150116). We used GBS for SNP discovery, since it is a high throughput and robust method even for species with complex genome and without reference genome [23]. The GBS method has already been used in alfalfa effectively [24]. The genetic linkage maps for each parent, 3010 and CW 1010 were constructed using JoinMap 5.0

[25].Linkage groups were assigned to each homologous chromosome using *Medicago* reference genome for both parental maps as described in [14,24].

The rust QTLs were detected for each parent using composite interval mapping (CIM) algorithm with Windows QTL Cartographer version 2.5 (Statistical Genetics, NC State University) as described previously [14]. A QTL was declared significant when the peak LOD value is > 3.0. The QTLs detected from the resistant parent CW 1010 were named Us-RustR1, Us-RustR2 etc. to represent *U. striatus* resistant QTLs. The QTLs mapped on the linkage map of the susceptible parent 3010 were referred to as Us-RustS1, Us-RustS2 and so on. The QTLs, their genomic locations and flanking markers were given (Table 4.3) with the sequences of the relevant peak markers of the QTLs (Table 4.4). The relationship between FD levels and RS as well as WH and RS of the F1 individuals were determined by estimating Pearson's correlation coefficient (r) (Table 4.2). We used dormancy and WH data taken over two consecutive years 2015/2016 and in winter of 2016/2017. The position of the QTLs detected with the highest R^2 value for each parental map were shown on the genetic maps using MapChart 2.3 (Figure 4.4) [26,14].

We conducted comparative analysis using the BLAST search of sequences of rust associated SNP alleles obtained from Tassel UNEAK (3.0) [27]. Sequences of the SNPs in the QTLs peak (Table 4.4) and flanking regions (not given) were used. The SNP sequences were searched against *Medicago* A17 reference genotype pseudomolecule assembly (Mt4.0) with BLASTn [28]. The best hit of BLASTn was opened using the genome browser (GBrowse) [29] to discern the alignment of alfalfa chromosome regions with the physical locations of putative homologs in *M. truncatula*.

Results

Phenotypic data analysis

The two parents showed different responses to the rust pathogen infection. The female parent 3010 had an average rust severity (RS) score of 4.0 (susceptible) while the male parent CW 1010 showed better resistance with a RS score of 2.3 (moderately resistant). Occurrence of pustules on the abaxial (Figure 4.2 left) and adaxial (Figure 4.2 right) surfaces of the rust infected leaves of both resistant and susceptible parents displayed clearly their response pattern. The pseudo-testcross F1 mapping population showed variable responses to rust and exhibited a nearly normal distribution (Figure 4.3), suggesting that the trait is most likely quantitative involving multiple loci that control resistance in alfalfa. The overall model was significant ($P < 0.01$) with a coefficient of determination (R^2) value of 0.65, which indicates a good fit of data to the linear model. There were significant differences among genotypes ($P < 0.01$) in their response to rust resistance. The mean rust score for the population was 3.2 and the LS means for the 184 F1 progenies ranged from 1.5 to 4.9. Transgressive segregants were present on both susceptible and resistant tails of the distribution (Figure 4.3).

Relationships between rust, dormancy, and winter hardiness

The two parents differ significantly in their FD with the resistant parent being non-dormant (FDR = 10) and the susceptible parent being very dormant (FDR = 2) and their progeny was segregating for dormancy [13]. Estimating the correlation of rust resistance with FD and WH of this population alfalfa would be valuable for the understanding of the disease incidence as well as the potential factors favoring it. The Pearson correlation coefficient (r) calculated for RS and FD was -0.26 ($P < 0.05$) and the coefficient (r) for RS and WH was -0.27 ($P < 0.05$), a possible indication that the more dormant (lower FD level) and winter-hardy (lower WH score)

alfalfa genotypes are more susceptible (higher rust score) to rust. This is the first report where dormancy, winter hardiness and alfalfa rust resistance were compared. Overlapping positions of QTLs detected for rust, dormancy and winter hardiness also indicated some relationship between these traits at least in this population.

QTL mapping of rust resistance

Five significant (LOD ≥ 3.0) QTLs (Us-RustR1, ……, Us-RustR5) associated with rust resistance were mapped on homologs 1B, 7A, 8A, 7C and 2C of the CW 1010 paternal linkage map, indicating that the inheritance of rust resistance in alfalfa is most likely polygenic (Table 4.3). The most important QTL identified on homolog 1B was detected at the LOD = 8.1 and had an R^2 = 0.13. The QTL peak of Us-RustR1 was located at the one LOD support interval (1-LSI) from 21.4 – 23.6 cM in WinQTL Cartographer. However, a larger segment of nearly 15 cM was covered by the entire QTL region, which was above the threshold LOD (> 3), indicating the genome region is important for rust resistance in alfalfa. This QTL Us-RustR1 shared the genomic location with a stable dormancy QTL ndorm8 reported previously [14] supporting the correlation between rust resistance and non-dormancy within the F1 (3010 x CW 1010) mapping population. Out of five rust QTLs detected for CW 1010, four had positive effects on rust resistance. An exception was the QTL Us-RustR2 with (R^2 = 0.11) (detected on homolog 7A of the resistant parent CW 1010, which had a negative effect on disease resistance (Table 4.3). The Us-RustR2 also overlaps in its genomic location with a potential dormancy QTL ndorm13 with negative effect [14]. Therefore, the QTL Us-RustR2 may represent an allele with negative effect on rust resistance and non-dormancy, although it was detected in the non-dormant and rust resistant parent CW 1010. Three rust QTLs that were mapped on homologs 8A, 7C and 2C of the CW 1010 genetic map had minor effect (R^2 = 0.05) on the rust phenotypic value (Table 4.3). The

QTL Us-RustR4 on homolog 7C also shares genomic location at 2-LSI with a stable dormancy QTL, ndorm2, reported previously [14]. The rust QTLs detected in this study are depicted on the genetic linkage maps with their one LSI and two LSI (Figure 4.4).

All rust QTLs mapped on the maternal (3010) genetic map showed negative effects on rust resistance. The QTL Us-RustS1 contributed the highest variation in the trait value with (R^2 = 0.08) and was detected at the LOD = 4.5 (Table 4.3). Similarly, other QTLs, Us-RustS2 and Us-RustS3 detected on homologs 8B and 3D accounted for 7% and 6% of the phenotypic variation, respectively. The 3010 QTL Us-RustS1 that was located on homolog 1A (91.7 – 94.2 cM) also co-localized with a stable dormancy QTL, dorm1, described in a previous report [14]. Therefore, in the F1 population (3010 x CW 1010), phenotypic and genetic relationship between dormancy and rust resistance were observed at least partly.

Furthermore, the QTLs for rust resistance also share genomic regions with WH QTLs reported before [14]. For instance, the rust QTL Us-RustR1 shares genomic regions with the QTL ws13 for WH QTL at 2-LSI [14]. The QTL Us-RustR2 on homolog 7A also resided very close to WH QTL ws4. Both Us-RustR2 and ws4 showed divergent effects (opposite direction) on rust (Table 4.3) and WH unlike other QTLs detected for CW 1010. Partial or complete overlapping of genomic regions of QTLs for RS and WH scores also was observed between Us-RustR3 and ws14, Us-RustR4 and ws1, and Us-RustS1 and wh1 [14], suggesting a potential genetic relationship between the biotic RS and abiotic WH in this alfalfa population.

Comparative analysis of rust QTL regions

Most of the SNP sequences subjected to BLASTn exhibited high similarities (~ 100 % identity) with segments of the *Medicago* genome. The output of BLASTn search was summarized in Table 4.5. The sequence of a flanking marker ‘TP83000’ of Us-RustR1 was

located in the proximity of a gene for cytidine/deoxycytidylate deaminase family protein in the *Medicago truncatula* reference genome. The other flanking marker 'TP7086' of the same QTL displayed homology with a *Medicago* gene for eukaryotic aspartyl protease family protein (Table 4.5). The peak and flanking markers 'TP27424' and 'TP51544' of Us-RustR2 best matched with a *Medicago* gene known as leucine zipper transcription factor. Similarly, a flanking marker 'TP46925' of Us-RustR3 showed homology to a gene for WEB family plant protein on *Medicago* genome. However, the marker TP46925 was mapped on the homolog 8A of alfalfa whereas the gene for WEB family plant protein is located on chromosome four of *Medicago*, evidencing a translocation between *Medicago* chromosome four and alfalfa chromosome eight as described previously [14]. Similarly, the sequence of a flanking marker 'TP54614' of Us-RustR4 displayed putative homology with the gene for pre-mRNA splicing factor-like protein, whereas the peak marker 'TP40466' of Us-RustR5 exhibited homology with a *Medicago* gene that codes for a cytosolic phosphoglycerate kinase (PGK) (Table 4.5).

The sequence of peak SNP 'TP78651' of Us-RustS1 detected in the susceptible parent 3010 displayed homology with a gene for phosphoglucomutase/phosphomannomutase family protein. The flanking marker 'TP15998' of Us-RustS1 revealed homology with *Medicago* glycerol-3-phosphate dehydrogenase gene (Table 4.5). Similarly, the peak marker 'TP75477' of Us-RustS2 QTL exhibited homology with a gene for a putative protein proton pump-interactor. The sequences of SNPs 'TP42683' and 'TP7327' in the flanking position of Us-RustS2 revealed homology with the gene for non-lysosomal glucosylceramidase and DUF630 family protein, respectively. Because of the reciprocal translocation between chromosome eight of alfalfa and four of *Medicago* [14], the SNP of Us-RustS2 were mapped on alfalfa chromosome 8B whereas the homologous segment in *Medicago* exists on chromosome four. The peak marker 'TP74002'

of QTL Us-RustS3 exhibited putative homology with a *Medicago* gene for Smad/FHA domain protein.

Discussion

This study reports the first QTL mapping effort for alfalfa rust resistance along with establishing homology between the sets of rust associated QTLs and putative homologous genes in the *Medicago truncatula* genome. A complex inheritance and incomplete resistance to rust in alfalfa was observed. In this study, five QTL mapped in the CW 1010 parent explained up to 38% ($R^2 = 0.38$) of the total phenotypic variation and the three QTLs detected in the 3010 parent accounted 21% ($R^2 = 0.21$) of the phenotypic variation. Although, relatively lower R^2 for disease QTL, yet enhancing up to 38% variation is important. The incomplete resistance to rust caused by various species of *Uromyces* was also reported for other cool season legume crops such as pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), chick pea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medik.) [30]. However, in other legumes such as soybean (*G. max* L.) and common bean (*Phaseolus vulgaris*) rust resistance is often controlled by one or a few major gene(s) [30,31]. Rubiales et al. (2013) described sources of incomplete or partial resistance to rust in lentil (*L. culinaris* Medik.) [32]. In the present study, we detected multiple QTLs with low to medium effects (Table 4.3) for both parental maps, which corroborates the polygenic inheritance of alfalfa rust resistance. Because of the use of only one specific type of GBS SNPs (single dose alleles) in the pseudo-testcross mapping, incomplete genome coverage or unsaturated maps might limit the detection of the whole set of loci controlling rust resistance. However, the average higher marker densities of both parental genetic linkage maps (1.5 cM/SNP) indicate that they were saturated enough for QTL detection [14]. Therefore, the probability of incomplete resistance to rust in alfalfa is very likely as is the case in some other cool season legumes.

Nevertheless, the need for extensive studies to characterize more sources of resistance to rust in alfalfa is suggested. The QTLs identified here may have potential for use in MAS to pyramid and increase the frequency of resistance alleles that could provide at least partial resistance to rust in alfalfa populations. Cloning QTLs with larger to smaller effects and pyramiding related genes may be the ideal way to enhance rust resistance in alfalfa [33]. Fine mapping of these QTLs may be essential to facilitate map-based cloning [34].

The moderate negative correlation between the phenotypic values of FD, WH, and alfalfa rust resistance/susceptibility observed in this study are a possible indication that dormant and winter-hardy germplasm might be more susceptible to rust. However, the relationship we observed here between traits could only be specific to the germplasm used rather than FD and WH in general. To draw a solid conclusion, this requires a more detailed investigation in a set of diverse alfalfa germplasm with different genetic backgrounds including dormant and non-dormant with different levels of winter hardiness. Nevertheless, some previous studies have shown that several common pathways such as signaling pathways take part in crosstalk between abiotic and biotic stresses [35]. For instance, previous research on rice reported that the Mitogen-activated protein kinase (MAPK) has positive effects on cold and other abiotic tolerance genes expression and subsequently the enzyme showed a negative effect on pathogenesis related gene expression [36]. This study also reported leucine zipper transcription factor as one of the homologs of rust related SNPs of alfalfa, which has also been reported as a regulatory gene for several biotic and abiotic stresses. For instance, the leucine zipper transcription factor regulates ABA-mediated stress response, salt and drought stress in tomato [37], lipid metabolism and cuticle biosynthesis in maize [38] and pathogen stress response in *Arabidopsis* [39]. Thus, molecules responding for one stress in plants may influence the pathways active for other

stresses. Therefore, the mechanisms of resistance to rust in alfalfa may be impacted by factors that also affect FD and WH. Furthermore, the cultivar 3010 which is adapted to winter hardiness zones in northern latitudes could have suffered more with *U. striatus* because of prevailing hot and humid weather of the Southeast, and the issue could be acclimation or adaptation. Further, rust infection that occurred in early November might have impacted the dormant but winter-hardy genotypes more as their foliage parts have been already weaker because of the translocation of carbohydrates and other reserves to their roots and crown parts for cold acclimation [40]. Since the resistance we observed was incomplete, it is possible that other physiological and cellular factors are at play in the variable response to *U. striatus* infections by the F1 individuals and their parents.

The best matches in sequence alignments of SNP sequences and the *Medicago* genome must be due to the evolutionary relationship between alfalfa and *Medicago*. This study identified several putative homologs for the SNPs under rust QTL which indicates the involvement of multiple molecular pathways in alfalfa resistance to rust. Furthermore, some of the *Medicago* genes/proteins identified here have previously been reported for their response to rust or other biotic and abiotic stresses. For instance, the eukaryotic aspartyl protease family proteins (Table 4.5) has been reported as an essential protein for defense against the fungal pathogen *Botrytis* in *Arabidopsis* [41]. Similarly, the homologs leucine zipper transcription factor, PGK, and FHA were previously recognized for their response to disease stresses in plants [39,42,43]. For example, the expression level of PGK changes in poplar leaf when it is infected with *Melampsora* rust fungus [44]. Therefore, the homologs reported in this study could be putative candidate genes for alfalfa breeding for *Uromyces* resistance.

In this study, we took advantage of evolutionary relatedness and synteny between alfalfa and *Medicago* genomes to locate putative homologs of rust SNPs and once again corroborate the importance of the model species *Medicago truncatula* for alfalfa genomic studies. However, *U. striatus* being a common pathogen, the results of this study would also be useful in searching for genomic regions underlying rust resistance in *Medicago*. The genetic maps of both species are highly syntenous [24] and have several conserved regions in both genomes. For instance, the CBF gene and its homologs for freezing tolerance are conserved between alfalfa and *Medicago*. However, the incomplete annotation of genes in the *Medicago* reference genome and the lack of a reference genome for *M. sativa* could hinder the identification of complete sets of candidate homologs. Moreover, a single QTL may have hundreds of genes [45] and here we BLAST searched only SNPs in peak and flanking regions, which would limit identifying some key rust regulating *Medicago* homologs.

Conclusion

In this study, a total of eight QTLs associated with leaf rust in alfalfa were detected that together explain up to 38% (R^2) variation in the phenotypic values. Since this is the first QTL work on alfalfa rust and the mapping was done in an F1 pseudo-testcross population, more investigations are required to explore the entire set of genetic factors involved in alfalfa rust resistance. This study suggests a polygenic inheritance and incomplete resistance to leaf rust in alfalfa. A negative correlation was observed between rust severity and FD levels, suggesting a possible genetic relationship between the dormancy state and leaf rust resistance, even though it is hard to rule out that the relationship may be cultivar specific. Similarly, a weaker negative correlation was also observed between rust and WH. The sequences of peak and flanking markers under QTL regions that aligned properly with *Medicago* reference genome Mt 4.0 A17

corresponded to putative homologs that may have an impact on regulating rust in alfalfa. The markers in QTL regions would be a valuable genomic resource to develop rust resistant alfalfa via MAS.

Tables and Figures

Table 4.1. Rating of rust severity (RS) on alfalfa mapping population infected with leaf rust in the field. Rating scores of 1-5 were assigned based on the NAAIC system.

Score	Severity Level	Disease signs and intensity
1	No disease infection	No visible infection and sign of disease on leaflet
2	Resistant	Flecks and closed pustules, 0-25% affected foliage
3	Moderately resistant	Some flecks closed and open pustules, 25-50% foliage affected
4	Susceptible	Several small open pustules, 50-75% foliage affected
5	Highly susceptible	Several large open pustules, 75-100% foliage get infected

Table 4.2. Relationships between fall dormancy (FD) and leaf rust severity (RS) score as well as winter hardiness (WH) and RS estimated using correlation analysis. The Pearson's correlation coefficient (r) of two compared variables were given. All correlations between the variables are statistically significant ($P < 0.01$).

Variables compared	Correlation coefficient (r)
FD assessed in Fall 2015 and RS score	-0.23
FD assessed in Fall 2016 and RS score	-0.25
Dormancy assessed in Winter 2016/2017 and RS score	-0.26
WH recorded in Winter 2015/2016 and RS score	-0.35
WH recorded in Winter 2016/2017 and RS score	-0.27

Table 4.3. Alfalfa leaf rust QTLs mapped in a resistant parent (CW 1010) and a susceptible parent (3010). The chromosome positions with relevant peaks and flanking markers along with their LOD and R^2 are indicated.

Parent	QTL code	Chr.	Peak Marker	Peak LOD	R^2	Allele Dir.	1-LSI (cM)	Flanking Markers
CW 1010	Us-RustR1	1B	TP41786	8.1	0.13	(+)	21.4 – 23.6	TP83000 - TP7086
CW 1010	Us-RustR2	7A	TP27424	7.5	0.11	(-)*	29.3 – 30.7	TP87998 - TP51544
CW 1010	Us-RustR3	8A	TP63006	5.6	0.05	(+)	19.3 – 19.7	TP68653 - TP46925
CW 1010	Us-RustR4	7C	TP38417	3.2	0.05	(+)	43.6 – 49.3	TP4972 - TP54614
CW 1010	Us-RustR5	2C	TP40466	3.0	0.04	(+)	29.8 – 38.7	TP40466 - TP70925
3010	Us-RustS1	1A	TP78651	4.5	0.08	(-)	91.7 – 94.2	TP995 - TP15998
3010	Us-RustS2	8B	TP75477	3.6	0.07	(-)	21.5 – 22.5	TP42683 - TP7327
3010	Us-RustS3	3D	TP74002	3.4	0.06	(-)	37.8 – 40.5	MRG_16533948 - TP1280

* Indicates a QTL with negative effect but detected in the rust resistant parent

Chr. = Chromosome

Dir. = Direction

Table 4.4. Alfalfa rust QTLs and the sequences of markers under QTL peaks. Two variant alleles for each SNP were denoted as 'query' and 'hit'. The SNP loci in the sequences are given in bold letters within a rectangle.

Parent	QTLs	Sequences for the SNPs indicated with bold letter	
CW 1010	Us-RustR1	>TP41786_query	CAGCTTCATAAATCTTTGAAGAAGTAGTAAAATCAGAATATTACTC**C**AAACAAAACCAGAAAGG
		>TP41786_hit	CAGCTTCATAAATCTTTGAAGAAGTAGTAAAATCAGAATATTACTC**T**AAACAAAACCAGAAAGG
CW 1010	Us-RustR2	>TP27424_query	CAGC**C**TGAAAGGGGGGAATCCAGTGAAAGATCAAAAGACAAATCGGATCAAAAGGTTATATAAT
		>TP27424_hit	CAGC**T**TGAAAGGGGGGAATCCAGTGAAAGATCAAAAGACAAATCGGATCAAAAGGTTATATAAT
CW 1010	Us-RustR3	>TP63006_query	CTGCATTTAAATATGGGTTTTTCTATTTTGTACCCATGCTAATCC**A**GCGCTAATTCAGTGTTAG
		>TP63006_hit	CTGCATTTAAATATGGGTTTTTCTATTTTGTACCCATGCTAATCC**G**GCGCTAATTCAGTGTTAG
CW 1010	Us-RustR4	>TP38417_query	CAGCTGAAGGACCTTCC**C**AAAAAAATGGAGGTAATACATTTTATGAGAACATTCAATAATCAGC
		>TP38417_hit	CAGCTGAAGGACCTTCC**G**AAAAAAATGGAGGTAATACATTTTATGAGAACATTCAATAATCAGC
CW 1010	Us-RustR5	>TP40466_query	C**A**GCTGTTGAGAAGGTTGGACTAGCAGACAAGATGAGTCACATCTCAACTGGTGGAGGTGCCAG
		>TP40466_hit	C**T**GCTGTTGAGAAGGTTGGACTAGCAGACAAGATGAGTCACATCTCAACTGGTGGAGGTGCCAG
3010	Us-RustS1	>TP78651_query	CTGCTATGTTAATAGAACCTCTTTGTATAATGCAGAAATAG**A**AGCGAAAGCATCAAATTATGAT
		>TP78651_hit	CTGCTATGTTAATAGAACCTCTTTGTATAATGCAGAAATAG**G**AGCGAAAGCATCAAATTATGAT
3010	Us-RustS2	>TP75477_query	CTGCTAAATCCTTATTATC**C**GATTTAGTTTTCAAATCTTTACCTTTGGTTTTGGTTTGTTTTTG
		>TP75477_hit	CTGCTAAATCCTTATTATC**T**GATTTAGTTTTCAAATCTTTACCTTTGGTTTTGGTTTGTTTTTG
30100	Us-RustS3	>TP74002_query	CTGCGGCGACTGAGACCTATGTGTGCCCCTTCC**A**TTGTGACGGTGCTCCGGCGACCTATCATGC
		>TP74002_hit	CTGCGGCGACTGAGACCTATGTGTGCCCCTTCC**G**TTGTGACGGTGCTCCGGCGACCTATCATGC

Table 4.5. Peak and flanking markers under one LSI of rust QTLs and their corresponding homologous genes in *Medicago truncatula* identified using BLAST search against Mt4.0 genome assembly.

Parent	Peak/Flanking Markers	Corresponding candidate homolog genes in *M. truncatula*	E-value	*M. truncatula* Homologs
CW 1010	TP83000	Cytidine/deoxycytidylate deaminase family protein	$1e^{-23}$	Medtr1g081550
CW 1010	TP7086	Eukaryotic aspartyl protease family protein	$4e^{-23}$	Medtr1g078240
CW 1010	TP27424	Leucine zipper transcription factor	$9e^{-25}$	Medtr7g089800
CW 1010	TP51544	Leucine zipper transcription factor	$9e^{-25}$	Medtr7g089800
CW 1010	TP46925	WEB family plant protein	$9e^{-25}$	Medtr4g478190
CW 1010	TP54614	Pre-mRNA splicing factor-like protein	$9e^{-25}$	Medtr7g068630
CW 1010	TP40466	Phosphoglycerate kinase-like protein	$3e^{-25}$	Medtr2g066130
3010	TP78651	Phosphoglucomutase/phosphomannomutase family protein	$9e^{-25}$	Medtr1g094980
3010	TP15998	Glycerol-3-phosphate dehydrogenase, putative	$3e^{-14}$	Medtr1g094185
3010	TP75477	Proton pump interactor, putative	$2e^{-21}$	Medtr4g132200
3010	TP42683	Non-lysosomal glucosylceramidase	$7e^{-26}$	Medtr4g134760
3010	TP7327	DUF630 family protein	$4e^{-23}$	Medtr4g127620
3010	TP74002	Smad/FHA domain protein	$9e^{-25}$	Medtr3g049440

Figure 4.1. A highly infected rust plant in the field at Watkinsville, Georgia. The mass of reddish-brown spores was clearly visible on foliage parts.

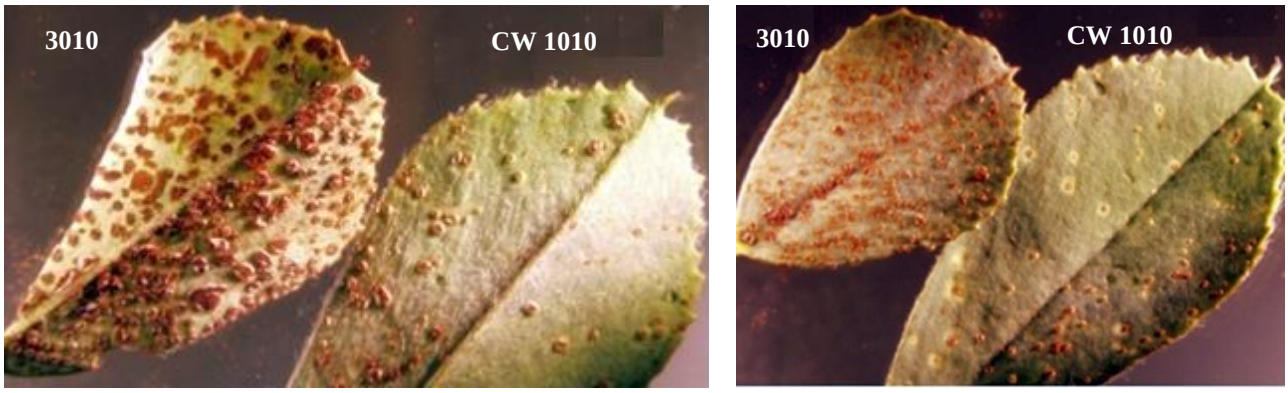


Figure 4.2. Magnified images showing abundance and sizes of rust pustules on the abaxial (left) and adaxial (right) surfaces of leaflets from the susceptible parent (3010) and the resistant parent (CW 1010).

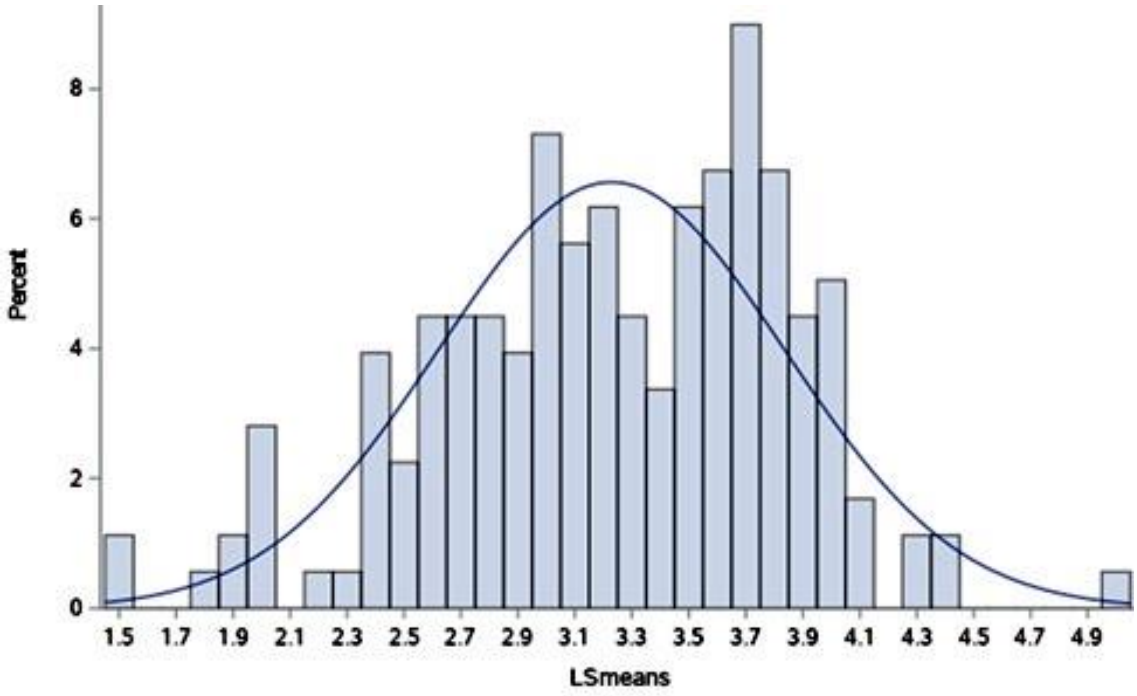


Figure 4.3. Distribution of rust infection scores of alfalfa F1 individuals rated from one (no infection) to five (highly susceptible). The bars represent the percentage of total plants with corresponding least square mean estimated for F1 rust scores at the X-axis.

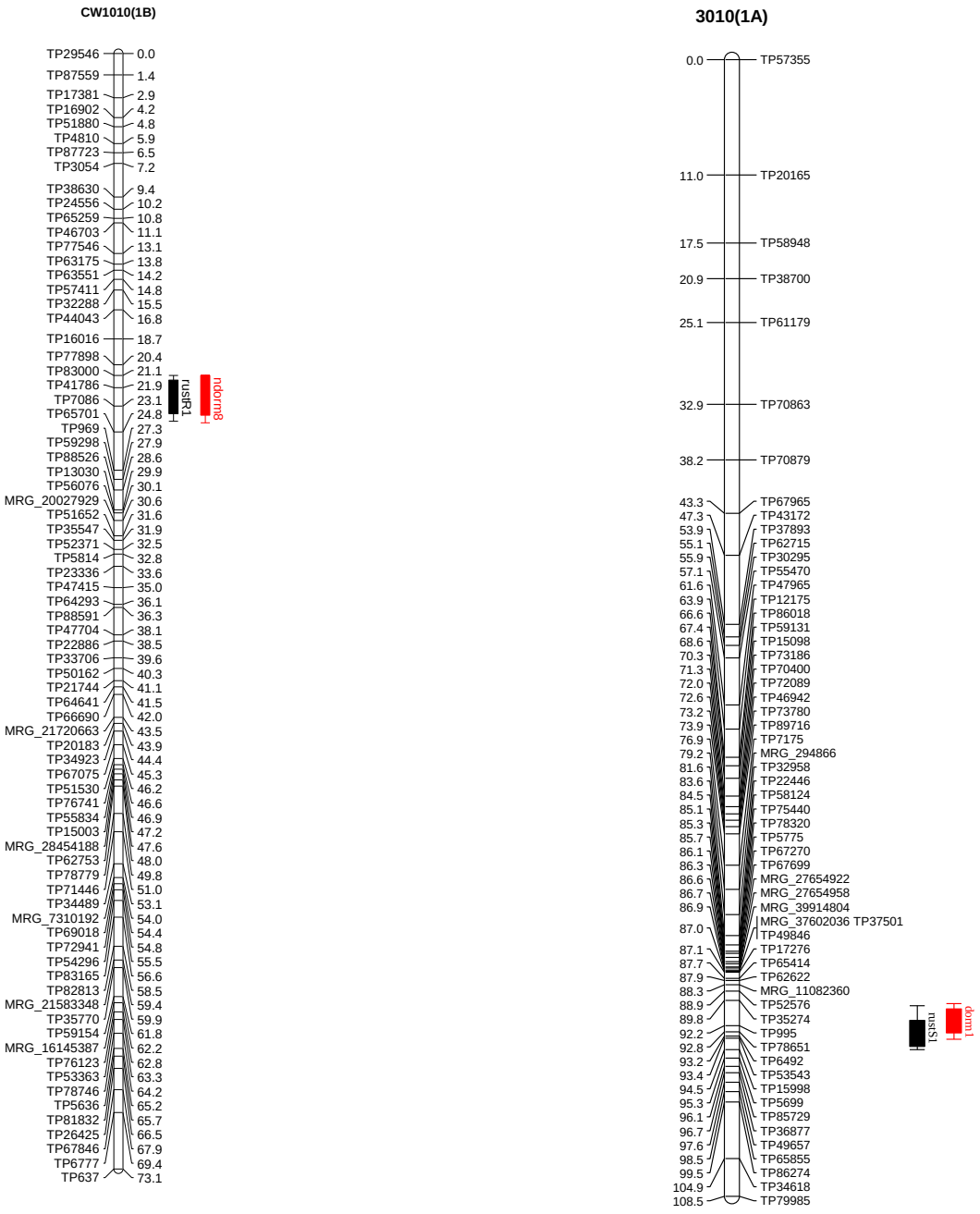


Figure 4.4. Linkage maps of homolog 1B of the paternal parent (CW 1010) (left), and homolog 1A of the maternal parent (3010) (right). The QTLs associated with rust (black bar) and their co-localization with previously identified dormancy QTL (red bar) are displayed. Since the two parents have contrasting dormancy levels, some rust QTLs mapped to the same genomic regions where dormancy QTLs were detected.

References

1. **Han Y, Kang Y, Torres-Jerez I, Cheung F, Town CD, Zhao PX, Udvardi MK, Monteros MJ** (2011) Genome-wide SNP discovery in tetraploid alfalfa using 454 sequencing and high resolution melting analysis. BMC Genomics 12:350-350. doi:10.1186/1471-2164-12-350

2. **Adhikari L, Missaoui AM** (2017) Nodulation response to molybdenum supplementation in alfalfa and its correlation with root and shoot growth in low pH soil. Journal of Plant Nutrition 40 (16):2290-2302. doi:10.1080/01904167.2016.1264601

3. **Webb D, Nutter Jr F** (1997) Effects of leaf wetness duration and temperature on infection efficiency, latent period, and rate of pustule appearance of rust in alfalfa. Phytopathology 87 (9):946-950

4. https://www.gov.mb.ca/agriculture/crops/production/forages/print,management-of-diseases-of-alfalfa-seed.html

5. http://alfalfa.ucdavis.edu/irrigatedalfalfa/pdfs/ucalfalfa8296disease_free.pdf

6. https://ipm.illinois.edu/diseases/rpds/301.pdf

7. service E-UAr (1960) Index of plant diseases in the United States. US Department of Agriculture

8. **Hanlin RT** (1966) Host index to the Basidiomycetes of Georgia. Georgia Experiment Station

9. http://corn.agronomy.wisc.edu/Management/pdfs/IPMManual_2_Alfalfa.pdf

10. **Webb D, Nutter Jr FW, Buxton DR** (1996) Effect of acid detergent lignin concentration in alfalfa leaves on three components of resistance to alfalfa rust. Plant disease 80 (10):1184

11. **Rubiales D, Moral A** (2004) Prehaustorial resistance against alfalfa rust (Uromyces striatus) in Medicago truncatula. European journal of plant pathology 110 (3):239-243

12. **Statler G** (1987) Alfalfa rust (Uromyces striatus) as a possible control of leafy spurge

13. **Samac DA, Foster-Hartnett D** (2012) Effect of glyphosate application on foliar diseases in glyphosate-tolerant alfalfa. Plant disease 96 (8):1104-1110

14. **Adhikari L, Lindstrom OM, Markham J, Missaoui AM** (2018) Dissecting Key Adaptation Traits in the Polyploid Perennial Medicago sativa Using GBS-SNP Mapping. Frontiers in Plant Science 9 (934). doi:10.3389/fpls.2018.00934

15. **Chu C-G, Friesen TL, Xu SS, Faris JD, Kolmer JA** (2009) Identification of novel QTLs for seedling and adult plant leaf rust resistance in a wheat doubled haploid population. Theoretical and Applied Genetics 119 (2):263-269. doi:10.1007/s00122-009-1035-0

16. **Garcia A, Calvo ÉS, de Souza Kiihl RA, Harada A, Hiromoto DM, Vieira LGE** (2008) Molecular mapping of soybean rust (Phakopsora pachyrhizi) resistance genes: discovery of a novel locus and alleles. Theoretical and Applied Genetics 117 (4):545. doi:10.1007/s00122-008-0798-z

17. **van Berloo R, Aalbers H, Werkman A, Niks RE** (2001) Resistance QTL confirmed through development of QTL-NILs for barley leaf rust resistance. Molecular Breeding 8 (3):187-195. doi:10.1023/a:1013722008561

18. **McIntyre CL, Hermann SM, Casu RE, Knight D, Drenth J, Tao Y, Brumbley SM, Godwin ID, Williams S, Smith GR, Manners JM** (2004) Homologues of the maize rust resistance gene Rp1-D are genetically associated with a major rust resistance QTL in sorghum. Theoretical and Applied Genetics 109 (4):875-883. doi:10.1007/s00122-004-1702-0

19. **Kemen E, Hahn M, Mendgen K, Struck C** (2005) Different resistance mechanisms of Medicago truncatula ecotypes against the rust fungus Uromyces striatus. Phytopathology 95 (2):153-157

20. **Vaz Patto MC, Rubiales D** (2014) Unveiling common responses of Medicago truncatula to appropriate and inappropriate rust species. Frontiers in Plant Science 5 (618). doi:10.3389/fpls.2014.00618

21. http://www.georgiaweather.net/index.php?variable=HI&site=WATUGA

22. https://www.naaic.org/stdtests/rust.pdf

23. **Buckler E, Glaubtiz J, Sun Q, Lu F, Harriman J, Mitchell S, Elshire R** (2012) Genotyping on High Throughput Sequencers: Preparation and Analysis of Reduced Representation Genomic Libraries. Journal of Biomolecular Techniques : JBT 23 (Suppl):S20-S20

24. **Li X, Wei Y, Acharya A, Jiang Q, Kang J, Brummer EC** (2014) A Saturated Genetic Linkage Map of Autotetraploid Alfalfa (Medicago sativa L.) Developed Using Genotyping-by-Sequencing Is Highly Syntenous with the Medicago truncatula Genome. G3: Genes|Genomes|Genetics 4 (10):1971-1979. doi:10.1534/g3.114.012245

25. https://www.kyazma.nl/docs/JM5Features.pdf

26. **Voorrips R** (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. Journal of heredity 93 (1):77-78

27. https://bytebucket.org/tasseladmin/tassel-5-source/wiki/docs/TasselPipelineUNEAK.pdf

28. **Ray I, Monteros M, Julier B, Sledge M, Brummer E** (2018) Identification of Consensus Regions Associated with Shoot Biomass Production in the Medicago Genome. Crop Science

29. http://www.medicagohapmap.org/fgb2/gbrowse/mt40/?name=chr4:30015038..30014975

30. **Rubiales D, Castillejo M, Madrid E, Barilli E, Rispail N** (2011) Legume breeding for rust resistance: lessons to learn from the model Medicago truncatula. Euphytica 180 (1):89-98

31. **Hurtado-Gonzales OP, Valentini G, Gilio TA, Martins AM, Song Q, Pastor-Corrales MA** (2016) Fine mapping of Ur-3, a historically important rust resistance locus in common bean. G3: Genes, Genomes, Genetics:g3. 116.036061

32. **Rubiales D, Rojas-Molina MM, Sillero JC** (2013) Identification of pre- and posthaustorial resistance to rust (Uromyces viciae-fabae) in lentil (Lens culinaris) germplasm. Plant Breeding 132 (6):676-680. doi:doi:10.1111/pbr.12096

33. **Soriano JM, Royo C** (2015) Dissecting the Genetic Architecture of Leaf Rust Resistance in Wheat by QTL Meta-Analysis. Phytopathology 105 (12):1585-1593. doi:10.1094/PHYTO-05-15-0130-R

34. **Sandal N, Krause K, Frantescu M, Radutoiu S, Krusell L, Madsen LH, Kanamori N, Madsen E, Tirichine L, Stougaard J** (2005) Mapping and map-based cloning. Lotus japonicus Handbook. Springer, pp 217-232

35. **Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K** (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Current Opinion in Plant Biology 9 (4):436-442. doi:https://doi.org/10.1016/j.pbi.2006.05.014

36. **Xiong L, Yang Y** (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid–inducible mitogen-activated protein kinase. The Plant Cell 15 (3):745-759

37. **Zhu M, Meng X, Cai J, Li G, Dong T, Li Z** (2018) Basic leucine zipper transcription factor SlbZIP1 mediates salt and drought stress tolerance in tomato. BMC Plant Biology 18:83. doi:10.1186/s12870-018-1299-0

38. **Javelle M, Vernoud V, Depège-Fargeix N, Arnould C, Oursel D, Domergue F, Sarda X, Rogowsky PM** (2010) Overexpression of the Epidermis-Specific Homeodomain-Leucine Zipper IV Transcription Factor OUTER CELL LAYER1 in Maize Identifies Target Genes Involved in

Lipid Metabolism and Cuticle Biosynthesis. Plant Physiology 154 (1):273-286. doi:10.1104/pp.109.150540

39. **Alves MS, Dadalto SP, Gonçalves AB, De Souza GB, Barros VA, Fietto LG** (2013) Plant bZIP Transcription Factors Responsive to Pathogens: A Review. International Journal of Molecular Sciences 14 (4):7815-7828. doi:10.3390/ijms14047815

40. **McKenzie J, Paquin R, Duke SH** (1988) Cold and heat tolerance. Alfalfa and alfalfa improvement (alfalfaandalfal):259-302

41. **Li Y, Kabbage M, Liu W, Dickman MB** (2016) Aspartyl Protease-Mediated Cleavage of BAG6 Is Necessary for Autophagy and Fungal Resistance in Plants. The Plant Cell 28 (1):233-247. doi:10.1105/tpc.15.00626

42. **Sekhwal MK, Li P, Lam I, Wang X, Cloutier S, You FM** (2015) Disease resistance gene analogs (RGAs) in plants. International Journal of Molecular Sciences 16 (8):19248-19290

43. **Feng B, Ma S, Chen S, Zhu N, Zhang S, Yu B, Yu Y, Le B, Chen X, Dinesh-Kumar SP, Shan L, He P** (2016) PARylation of the forkhead-associated domain protein DAWDLE regulates plant immunity. EMBO Reports 17 (12):1799-1813. doi:10.15252/embr.201642486

44. **Major IT, Nicole M-C, Duplessis S, Séguin A** (2010) Photosynthetic and respiratory changes in leaves of poplar elicited by rust infection. Photosynthesis Research 104 (1):41-48. doi:10.1007/s11120-009-9507-2

45. **Gelli M, Mitchell SE, Liu K, Clemente TE, Weeks DP, Zhang C, Holding DR, Dweikat IM** (2016) Mapping QTLs and association of differentially expressed gene transcripts for multiple agronomic traits under different nitrogen levels in sorghum. BMC Plant Biology 16 (1):16. doi:10.1186/s12870-015-0696-x

CHAPTER 5

RELEVANCE OF INDOOR FREEZING TOLERANCE TESTING TO PREDICT ALFALFA WINTER HARDINESS

Introduction

Low-temperature in winter affects alfalfa (*Medicago sativa* L.) growth and development leading to reduced biomass yield and stand persistence [1]. Severe winter damage in alfalfa is frequent in northern climate [2,3]. Improvement of alfalfa for winter hardiness (WH) has traditionally been achieved via mass and recurrent selection of superior genotypes in field nurseries [4]. However, selection for cold hardiness in nurseries is often low efficient because of the winter seasonal variations and requirement for data from multiple years in multiple locations. [4]. In addition, accurate cold hardiness characterization in plants necessitates data records for several dates in a season, thereby making the process costly and laborious [5]. The NAAIC protocol for winter survival rating recommends data for a minimum of two locations and years to overcome possible year/location variation in the nature of winter injury [6]. NAAIC also recommends some precautions that should be considered in the collection and interpretation of alfalfa winter survival data. For instance, the winter survival ratings recorded too early can underestimate the WH of dormant genotypes. Therefore, assessment of WH in the field is relatively tedious and a long process. Further, alfalfa selection against winter damage using conventional breeding is very slow as the trait has quantitative inheritance and experiences substantial interaction with the environment [7].

Freezing tolerance is an important factor for predicting winter hardiness in a field as freezing injury (FI) and winter injury (WI) are positively correlated [8]. Evaluation of plant's ability to tolerate freezing temperatures in the lab with simulated environment has been considered an efficient and rapid technique to identify superior cold hardy genotypes for breeding. Such lab tests can be performed using a traditional temperature bath [9], freezer chambers with programmed temperatures [4], as electrolytic leakage, and as chlorophyll fluorescence assays. Nevertheless, a critical question arises; does the lab tested alfalfa outperform winter susceptible alfalfa during harsh winters in the field? Several studies have tried to explain the relationship between laboratory tests and nursery performance of plants against cold stress. Adkins et al. (2002) evaluated cold hardiness of ten species of the genus *Hydrangea* in the lab, where they observed the performances of *H. macrophylla* cultivars corresponding to their landscape performance reported in earlier studies [10]. A strong positive correlation between freeze test and plant cold hardiness visual ratings were reported for St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] [11]. Stable performance of plants in the nursery and artificial environment of indoor cold selection is likely due to common physiological, biochemical and cellular variables.

Low-temperature induces several genes and cold signal transduction pathways generating required biomolecules (proteins, soluble sugars, osmo-protectants) for cold acclimation. For instance, the *CBF* (C-repeat binding factor) gene family, the most important genes controlling cold acclimation in plants, is induced under cold environment regardless of the source of temperatures [12]. In fact, cold sensitive plants often lack the signaling molecules or associated genes or regulatory molecules by mutation or by gene inactivation and fail to be cold acclimated. Other factors such as water stress, pesticide application, fertilizer treatment, bacterial

colonization, and planting date [9] also affect plant hardiness against cold in the field. Nevertheless, the major cellular changes in plants arise only as a result of low-temperature. Therefore, regardless of the experimental sites and source of temperature, sensitive plants exhibit cold injuries under low temperatures.

In the Southeast USA, winters have occasional frost and fluctuating temperatures. This kind of environment can be damaging to non-dormant alfalfa. When winters are warm with temperature near 13 °C, over-wintering alfalfa break dormancy and start new growth with elongated crown buds [1]. The process depletes alfalfa root reserves, which eventually makes the plant cold susceptible to low-temperature [1]. Therefore, cold hardy alfalfa genotypes are desirable in the region to protect alfalfa from winter damage. In alfalfa, freezing tolerance was enhanced by up to 5 ˚C in progeny of indoor selected superior parents, which eventually increased WH of the population [4]. Indoor low-temperature selection also enables screening a large number of germplasms in a relatively shorter period [4]. This method may also be cheaper and faster to enhance alfalfa winter hardiness and expedite cultivar improvement. Therefore, the objectives of this experiment were i) to optimize a protocol for alfalfa freezing tolerance test in a freezing chamber for indoor screening, and ii) to estimate the relationship between alfalfa indoor freezing tolerance and winter hardiness assessed in the field.

Materials and Methods

This experiment was conducted to test freezing tolerance of alfalfa pseudo-testcross F1 progeny (3010 (♀) x CW 1010 (♂)) developed for QTL mapping of various adaptations and agronomic traits. The QTL mapping population development and experimental design in the field were described previously [13]. We were able to complete freeze testing in 78 genotypes out of the 184 F1 population. The research will be resumed with a new ESPEC (ESPEC North

America, Inc.) freezer. Ten clones generated by stem-cuttings from each genotype were tested for freezing tolerance.

Before testing the F1 plants, we optimized a protocol for freezing test using alfalfa standard check cultivars for winter survival (WS). There are six WS checks recommended by NAAIC, including ZG 9830 (WS = 1), 5262 (WS = 2), WL325HQ (WS = 3), G-2852 (WS = 4), Archer (WS = 5), and Cuf 101 (WS = 6) [6]. The checks seed/clones were sown/transplanted in 14 cm cone-tainers filled with farm soil and 5 cm depth of the germ mix at the top of the cone. The cones were placed in RL98 trays.

The clones were grown in the greenhouse for 6-8 weeks. Then, the plants were transferred to an acclimation chamber at a temperature of 4 °C for 3 weeks. The chamber was adjusted to eight hours of light/16 hours dark period, and 70% relative humidity (RH). The plants were watered weekly, and Hoagland's nutrient solution was applied once during the acclimation period. The treatment and control sets, each on a separate RL98 tray, were acclimated as a single test. Various combinations of cold temperature and time of exposure were tested to optimize the freezing trial until significant differences, using chi-square test ($P = 0.95$), were observed between samples of the checks 5262 (WS=2) and G-2852 (WS = 4), as described previously [6]. After the cold treatment, the plants were moved to normal greenhouse conditions. The top portion of the plants for both the control and the treatment group samples were then clipped, leaving two nodes above the crown to allow regrowth. After two weeks of treatment, the data was recorded as survival percentage, regrowth height, and visual rating of injury on a scale 1-5. The visual rating procedure was described in our previous study [13]. Biomass of plants in both treatment and control groups were taken after three weeks of the freezing test. The data from the indoor freezing test was compared with WH data recorded in the field at two locations,

Watkinsville (JPC farm) and Blairsville (BVL), GA using Pearson's correlation coefficient (r). The PROC CORR procedure in SAS 9.4 (SAS Institute Inc.) was used to estimate the correlation. In 2017, WH data in the field was collected after late season frost in early March, where winter injury was very obvious [13]. We analyzed the correlation between indoor freezing test data and 2017 WH field data. The indoor freezing test data was also compared with overall WH data from three years at both field-testing locations.

Results

Control and treatment sets of the standard check cultivars 5262 (WS = 2) and G-2852 (WS = 4) showed significant differences ($P < 0.05$) when the plants were exposed to freezing temperature of -8 °C (Figure 5.1). The optimized protocol included a series of combinations of temperatures and durations of exposure. Both treatment and control plant sets were maintained at 0 °C for 8 hours. Then, the control set was removed from the freezing chamber and transferred to normal growing conditions at 14 hr. of light (23 °C) and 10 hr. of dark (15 °C). Plants in the treatment sets were maintained at below freezing temperature in a way that the temperature was decreased by 2 °C/hr until it reached -8 °C. The plants were maintained at the temperature -8 °C for 90 min, then the temperature was raised gradually by 2 °C/hr until it reached 2 °C. The plants from both control and treatment were then transferred to the normal greenhouse condition as mentioned above and kept for 3 weeks for regrowth and biomass data.

Segregation in the F1 progeny and phenotypic correlations

We observed variation in the 78 F1 genotypes tested for cold temperature tolerance. The percentage of survival (PS) of cold treated genotypes ranged from 7% to 100%. Mean regrowth height ratio (RHR) of surviving plants in treatment and control ranged from completely sensitive genotypes (near 0) to almost completely tolerant genotypes (near 1). Similarly, the mean biomass

ratio (BR) of surviving plants in treatment and control sets ranged from 0.01 to 0.97. The average visual rating (VR) of freezing tested genotypes varied from 1 to 4.9, while the ratio of VR of treatment and control (VRR) ranged from 1 to 4.8 indicating that variability is present in the F1 genotypes for low temperature sensitivity. The genotypes with higher regrowth height ratio (RHR) and biomass ratio were considered tolerant to freezing temperature.

The PS showed strong negative correlations ($r = -0.91$, $P \leq 0.01$) with mean VR, which means higher % of the surviving clones indicating better cold tolerance (low VR) (Table 5.1). The VRR also had similar strong negative correlation with the mean PS. The significant positive correlations ($P \leq 0.01$) between PS and RHR, and PS and BR indicated that the genotypes with higher survival percentage produced higher regrowth and subsequently higher biomass. Strong negative correlations were also observed between the variables VR and RHR as well as between VRR and RHR, suggesting that the more cold sensitive genotypes (high VR and VRR) had a low regrowth. Similarly, significant negative correlations ($P < 0.01$) were obtained between BR and VR as well as between BR and BRR with moderate r values (Table 5.1).

We found significant positive correlations ($r = 0.36$, $P < 0.01$) between mean VR and the LS means of WH scores collected at the JPC field location in 2017 (WH017JPC). A significant positive correlation ($r = 0.26$, $P < 0.01$) was also observed between VR and WH data from the Blairsville location in 2017 (WH017BVL). The VRR of indoor screened samples also exhibited positive correlations with WH data of both environments overall years (WH-JPC, and WH-BVL) (Table 5.1). This study reveals that indoor screening of alfalfa plants for cold temperature tolerance is relevant to the selection of alfalfa accessions for WH. Besides VR, other variables, such as PS, RHR and BR, from indoor testing also displayed significant correlations ($P < 0.05$) with WH data from the field at the JPC location (Table 5.1). However, we could not find

significant correlations ($P < 0.05$) between field data for BVL and the variables PS, RHR and BR. But, the direction of the relationship between them was similar to that exhibited by the JPC field data.

Discussion

In this experiment, we optimized a protocol for indoor screening of alfalfa in below freezing-temperature tolerance, which could be useful to select alfalfa for enhanced cold hardiness. Nevertheless, WH is a broader term that refers to plant's ability to withstand harsh winters, which is often encompass freezing temperature, diseases, high moisture level, ice formation, and frost-heaving [14]. In a study performed to determine the differences between winter hardy and non-winter hardy alfalfa in terms of carbohydrate accumulation in the crown, artificial freezing and natural hardening found different levels of carbohydrate accumulations in crowns [15]. Alfalfa freezing tolerance was more relevant to accumulation of sucrose, stachyose and raffinose and decreased levels of glucose, fructose and starch, whereas in natural hardening (maximum freezing tolerance) the crowns accumulate more raffinose and stachyose, and less relevant to sucrose accumulation [15]. Freezing tolerance is one of the predictors of alfalfa WH [8].

Brouwer et al. (2000) found a positive correlation ($r = 0.34$ to 0.58) between freezing injury and winter injury in the field condition, suggesting that freezing tolerance and WH are potentially controlled by the same genetic mechanism [8]. Castonguay et al. (2009) used cold tolerant alfalfa from indoor selection to develop breeding populations, which accelerated the breeding process [4]. In alfalfa, recurrent phenotypic selection is still a widely accepted method for WH selection [4]. For instance, superior freeze tolerant cultivars like Apica (ATF0) and (ATF5) were developed using recurrent selection for up to five cycles [16]. In our indoor cold

tolerance test, we found a positive correlation (up to $r = 0.36$, $P < 0.05$) between alfalfa freezing tolerance rating (VR) and WH scores recorded in the field. As young clonal seedlings were tested in a cold chamber while plants in the field were already rooted for years, the differences in the age of the plants tested in the two conditions may be the reason for the lower correlation between VR and WH than expected. The results indicate that indoor cold tolerance testing of alfalfa can be used in selecting for alfalfa winter hardiness, but selection solely based on indoor cold tolerance may not be sufficient. Testing indoor selected plants in the field is recommended because freezing tolerance is only a component of WH, and no perfect simulation environment can be created in an indoor chamber, which essentially controls just the temperature.

Furthermore, winter injury in the field is more noticeable under fluctuating temperatures, and the data were collected in the field after frost occurrence. While in the indoor chamber, we constantly decreased and subsequently increased the temperature with no fluctuations. In other words, alfalfa becomes cold sensitive either by not exhibiting cold acclimation initially or by losing the originally attained acclimation through de-acclimation. Past reports indicated that when temperature in winter rises to a mild range (~ 13 °C), the alfalfa plants begin deacclimation, loss/reduce hardiness attained via acclimation, and initiates new buds [1]. When the mild temperature goes down, winter hardy plants exhibit re-acclimatization. The genotypes that fail to exhibit re-acclimation (winter susceptible) will suffer from winter injury [12]. Therefore, our indoor screening method could be more effective, if we created the conditions of acclimation, deacclimation and reacclimation.

One of the challenges we encountered in the alfalfa indoor cold test was to prepare identical stem cutting-clones. The clonal variation is not unusual and such variation among cuttings of the same genotype were commonly experienced in alfalfa. Perhaps the uncontrolled

source of variability is attributable to the vigor of establishment and the interaction with the environment (watering, location in the growth chamber, etc.) (Yves Castonguay, personnel communication, 2016). This effect can be minimized by making multiple clonal propagules for each genotype and using the vigorous clones of uniform size for cold treatment. When resources are not limited, producing a large number of cuttings and testing more uniform clones could be effective to avoid the uncontrolled source of variability among clones. However, the phenotypic variation present in clones, which is also known as somaclonal variation, could be the result of other factors such as epigenetic changes [17].

Conclusion

Plants growing under natural conditions must cope with several environmental challenges to ensure survival. For alfalfa, winter damage is one of the environmental factors limiting its production, especially in cold climates. Low and non-freezing temperatures and occasional frost can injure alfalfa plants and impact their productivity, survival, and ecological adaptation. Therefore, improvement of alfalfa for cold tolerance is essential to minimize winter damage. In this study, we observed that alfalfa WH can be enhanced using surrogate phenotype selected for cold hardy genotypes under artificial cold environment. This indirect selection can be recommended to supplement the direct field selection for WH because we observed a significant positive correlation ($P < 0.05$) between cold tolerance and WH rating. Therefore, the indoor cold selection method can be useful to accelerate the breeding process of improving alfalfa for WH.

Tables and Figures

Table 5.1. Correlations between different variables from indoor freezing tolerance testing and winter hardiness data recorded under field conditions.

	PS	RHR	BR	VR	VRR	WH017JPC	WH-JPC	WH017BVL	WH-BVL
PS		0.55^{***}	0.40^{**}	-0.91^{***}	-0.70^{***}	-0.33^{**}	-0.26^{*}	-0.23^{NS}	-0.19^{NS}
RHR			0.78^{***}	-0.65^{***}	-0.75^{***}	0.19^{NS}	-0.25^{*}	-0.22^{NS}	-0.25^{NS}
BR				-0.46^{***}	-0.64^{***}	-0.25^{*}	-0.28^{*}	-0.20^{NS}	-0.13^{NS}
VR					0.80^{**}	0.36^{**}	0.25^{*}	0.23^{*}	0.24^{*}
VRR						0.36^{**}	0.26^{*}	0.16^{NS}	0.15^{NS}

* $P < 0.05$, **$P < 0.01$, *** $P < 0.001$, ^{NS} non-significant

Table abbreviations

PS = Percentage survival

RHR = Mean regrowth height ratio of surviving plants (treatment/control)

BR = Ratio of mean wet biomass of surviving plants (treatment/control)

VR = Mean visual rating of low-temperature tolerance of a cold treated genotype

VRR = VR ratio of treatment and control groups

WH017JPC = Winter hardiness least square mean, data collected in year 2017 at JPC

WH-JPC = Winter hardiness least square mean, overall (3 years) data at JPC

WH017BVL = Winter hardiness least square mean, data collected in 2017 at Blairsville

WH-BVL = Winter hardiness least square mean, overall (3 years) data at location Blairsville

Figure 5.1. Regrowth pattern of indoor freeze tested alfalfa plants from the treatment group (left) and the control group (right) after two weeks of treatment.

References

1. http://www.canr.msu.edu/uploads/resources/pdfs/e2310.pdf

2. **Leep R, Andresen J, Jeranyama P** (2001) Fall Dormancy and Snow Depth Effects on Winterkill of Alfalfa, vol 93. doi:10.2134/agronj2001.9351142x

3. **Bélanger G, Castonguay Y, Bertrand A, Dhont C, Rochette P, Couture L, Drapeau R, Mongrain D, Chalifour F, Michaud R, A D, P C, Chalifour D** (2006) Winter damage to perennial forage crops in eastern Canada: Causes, mitigation, and prediction, vol 86. doi:10.4141/P04-171

4. **Castonguay Y, Michaud R, Nadeau P, Bertrand A** (2009) An indoor screening method for improvement of freezing tolerance in alfalfa. Crop science 49 (3):809-818

5. **Lindstrom O, Dirr M** (1989) Acclimation and low-temperature tolerance of eight woody taxa. HortScience 24 (5):818-820

6. https://www.naaic.org/stdtests/wintersurvivalnew.pdf

7. **Castonguay Y, Dubé M-P, Cloutier J, Bertrand A, Michaud R, Laberge S** (2012) Molecular physiology and breeding at the crossroads of cold hardiness improvement, vol 147. doi:10.1111/j.1399-3054.2012.01624.x

8. **Brouwer DJ, Duke SH, Osborn TC** (2000) Mapping Genetic Factors Associated with Winter Hardiness, Fall Growth, and Freezing Injury in Autotetraploid Alfalfa Research support provided by a Hatch grant from the College of Ag. and Life Sci., Univ. of Wisconsin-Madison. Crop Science 40 (5):1387-1396. doi:10.2135/cropsci2000.4051387x

9. **Lindstrom OM** (1992) Transplant date influences cold hardiness of Leyland cypress following transplanting into the field. HortScience 27 (3):217-219

10. **Adkins JA, Dirr MA, Lindstrom OM** (2002) Cold hardiness potential of ten Hydrangea taxa. Journal of Environmental Horticulture 20 (3):171-174

11. **Kimball JA, Tuong TD, Arellano C, Livingston Iii DP, Milla-Lewis SR** (2017) Assessing freeze-tolerance in St. Augustinegrass: temperature response and evaluation methods. Euphytica 213 (5):110. doi:10.1007/s10681-017-1899-z

12. **Hincha DK, Zuther E** (2014) Plant Cold Acclimation:6. doi:10.1007/978-1-4939-0844-8

13. **Adhikari L, Lindstrom OM, Markham J, Missaoui AM** (2018) Dissecting Key Adaptation Traits in the Polyploid Perennial Medicago sativa Using GBS-SNP Mapping. Frontiers in Plant Science 9 (934). doi:10.3389/fpls.2018.00934

14. https://extension.umaine.edu/publications/2272e/

15. **Castonguay Y, Nadeau P, Lechasseur P, Chouinard L** (1995) Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winterhardiness. Crop Science 35 (2):509-516

16. **Dubé M-P, Castonguay Y, Cloutier J, Michaud J, Bertrand A** (2013) Characterization of two novel cold-inducible K3 dehydrin genes from alfalfa (Medicago sativa spp. sativa L.). Theoretical and Applied Genetics 126 (3):823-835

17. **Pikaard CS, Mittelsten Scheid O** Epigenetic regulation in plants. Cold Spring Harbor perspectives in biology 6 (12):a019315-a019315. doi:10.1101/cshperspect.a019315

CHAPTER 6

SUMMARY

Dissecting the genetic basis of alfalfa major adaptation and agronomic traits is valuable for understanding the adaptation mechanisms and improving these traits to increase yield and persistence. The presence of phenotypic variation for two important adaptation traits, fall dormancy (FD) and winter hardiness (WH) in alfalfa germplasm provides opportunity for breeders to integrate these important attributes for improving alfalfa to overcome production limitations associated with environmental changes. Incorporation of WH in non-dormant alfalfa germplasm requires understanding of the genetic basis of both WH and FD. Winter-hardy non-dormant alfalfa cultivars would fit in a wider adaptation range and have extended growing season with additional harvests that would reduce seasonal forage gaps. Seasonal forage gaps exist because of partial to complete growth cessation of warm-season species when cool-season forages are not productive yet. Winters in Georgia and the southeast USA are overall mild but experience a few freezing days per season depending on the latitude and elevation. These regions with mild winters can be cultivated with winter-hardy non-dormant alfalfa germplasms that provide additional cuttings and extend the forage production season. Furthermore, if the selected winter-hardy and non-dormant alfalfa cultivars carry favorable alleles for other important agronomic traits such as early spring yield (SY), timing of flowering (TOF), late summer yield and disease resistance, they will ensure year-round alfalfa production with high-quality biomass. Therefore, this project was carried out with the objective to understand the genetic basis of the variations present in an alfalfa population for the traits of interests (FD, WH, TOF, SY, summer

yield and rust resistance) using quantitative trait loci (QTL) mapping in a pseudo-testcross population. Another objective of the project was to optimize a protocol for alfalfa cold tolerance in an indoor freezer and to test the protocol in an alfalfa mapping population.

In alfalfa, identifying genomic regions controlling important phenotypes is hindered by the lack of a physical genome map and limited availability of polyploid friendly genomic tools. With the advent of Next Generation Sequencing (NGS) and cost effective NGS library preparation approaches, such as genotyping-by-sequencing (GBS), discovery of SNP markers has become easier in species even without reference genomes. However, alfalfa exhibits tetrasomic inheritance and only markers segregating in specific patterns can be used for genetic mapping with the available genetic mapping tools. We used single dose allele (SDA) markers unique to each parent for genetic mapping. Markers segregating in a single parent are also known as testcross markers, and the genetic mapping strategy using such markers is known as pseudo-testcross strategy, which was used for this project. This is a common approach for genetic mapping of a population derived from two out-crossing parents.

This study utilized the GBS method for SNP identification and genotyping of an F1 mapping population derived by crossing two alfalfa cultivars, 3010 (♀) x CW 1010 (♂). The two parents had contrasting FD and WH, with 3010 being dormant, winter-hardy and CW 1010 being non-dormant, winter susceptible. A total of 184 F1 population was developed and replicated using stem cuttings. The F1 clones, two parents, and check cultivars were transplanted at two locations, Watkinsville and Blairsville, GA. The Blairsville location was selected because the winters in this location are often harsh with freezing temperature and frequent snow. The two locations were also different from each other in other climatic variables, such as the year-round

temperature and rainfall, which allowed us to understand the environmental interaction of the traits of interests.

For FD phenotyping, regrowth height of F1 progeny, standard checks, and parents was measured at four weeks after clipping on 21st September. As sufficient regrowth was observed in the winter (2016-2017), early winter and late winter height data were also recorded to see if it is necessary to adjust the North American Alfalfa Improvement Conference (NAAIC) dormancy protocol for the mild winters of the southeast environments. We established regression equations, for each season dataset, using regrowth heights of FD standard checks and their dormancy rating in the field. Then, the FD of mapping population were estimated using their height data in the equation as the independent (predictor) variable. WH was assessed using visual rating scores (0-5), with zero being no winter effect, one being the most winter-hardy and 5 being the most winter susceptible. Data for WH was recorded in the winter months. Visual rating of winter damage was recorded after each freezing occurrence or monthly basis (in the case of a mild-winter). Alfalfa TOF was assessed after the onset of spring by estimating days to flowering when all four clones within a plot have at least one flower using the Julian calendar days. The F1 plants were clipped after completion of flowering in spring and the wet biomass was recorded as SY. After the first spring harvest, we also recorded biomass yield from three subsequent summer cuts and analyzed it as cumulative summer biomass (CSB). Segregation of the mapping population for its response to rust infection allowed for the evaluation of alfalfa rust severity using a visual rating scale 1 to 5. We set up a protocol for testing alfalfa clones in an artificial freezing environment in an ESPEC chamber using WH checks. The freezer tested alfalfa plants were phenotyped using regrowth height and biomass ratios of treated vs. control groups and

visual ratings. The phenotypic correlations between the various traits considered in this study were estimated using Pearson's correlation coefficient (r).

The molecular data was generated using genotyping-by-sequencing (GBS). Raw reads from GBS were processed using two pipelines, UNEAK and GBS-SNP-CROP that helped us to discover 5322 single dose allele (SDA) markers unique to the maternal parent (3010) and 2327 SDA SNPs for paternal parent (CW 1010). The SDA SNPs from each parent were mapped using the pseudo-testcross method and JoinMap 5.0. The 32 linkage groups of each parent were assigned to eight chromosomes, each with four sub-genomes, using reference genome (Mt4.0) of *M. truncatula*. The constructed genetic linkage maps were used for QTL detection using composite interval mapping algorithm in Windows QTL cartographer (2.5). The least square (LS) means of the phenotypic data of each trait for each genotype were estimated using PROC GLM in SAS 9.4, and the LS means of F1 were used as the trait value for QTL mapping.

In this study, we observed important phenotypic correlations among several alfalfa traits. A moderate positive (0.22 - 0.57) correlation was observed between FD and WH rating, whereas the correlation magnitude varied greatly with FD assessment time. FD assessed based on regrowth data after clipping alfalfa on 21st September showed weaker correlation with WH, whereas the dormancy assessed in winter exhibited stronger relationships with WH. The effect of dormancy in the alfalfa population was more visible in mild winters. Therefore, we recommend to clip alfalfa later in late fall or early winter to obtain reliable regrowth height data to assess FD dormancy effectively in regions like Georgia with warm late fall temperatures.

We also observed phenotypic correlations between traits such as TOF and FD, WH and SY, and rust severity with FD and WH. These correlations suggest potential cross-talk between various alfalfa traits. However, the relationship may be specific to the particular population and

further studies in diverse alfalfa populations may be needed. Nonetheless, the study reveals the direction of relationship between traits. Understanding the phenotypic relationship among traits is important for developing more plastic alfalfa cultivars with expanded adaptability, higher yield, and other essential characteristics. The moderate positive correlations observed between indoor cold screening and field WH rating indicated that WH selection in the field can be accelerated by screening alfalfa initially in freezing chambers.

To our knowledge, this is the first report on QTL mapping in alfalfa at sub-genome level using dense genetic linkage maps. The genetic maps for both parents had an average marker density of 1.5 cM/SNP. Several novel QTLs including stable QTLs and evolutionarily conserved QTLs were identified for the important alfalfa traits in this study. Forty-five QTLs for FD and 35 for WH were detected on both male and female linkage maps. The QTLs were identified mostly in the separate genomic regions, which indicates that the two traits are genetically separate, and their independent improvement is possible. We also detected few overlapping (matched) QTLs for these two traits. The overlapping QTLs and the positive phenotypic correlations observed between FD and WH could be due to the presence of pseudo-linkages because of long-term simultaneous selection for those two adaptation traits. This study provides insights into the relationship between seasonal dormancy and WH, which can also be applied to other perennials.

In this study, a total 25 QTLs for TOF, 17 for SY and six QTLs for CBS biomass were detected. Three TOF related QTLs were found stable and four TOF QTLs were detected at the position relative to the flowering QTLs of *M. truncatula* identified previously. Several putative homologous genes for the SNP sequences of QTLs peak and flanking regions were observed for TOF, SY and CBS. This study provides the first QTL mapping analysis for rust resistance in alfalfa. We detected eight alfalfa QTLs of rust resistance and the QTLs that explained maximum

variation in phenotype had $R^2 = 0.13$. The experiment clearly showed that leaf rust resistance in alfalfa is polygenic.

Further, the QTLs and molecular markers associated with the variation in alfalfa phenotypes investigated in the study will add genomic resources and enable marker-assisted selection (MAS) to speed up the development of non-dormant alfalfa cultivars with enhanced WH. The non-dormant, winter-hardy and widely adapted alfalfa cultivars with an extended growing season can enhance overall forage production and fill seasonal gaps. Being the most widely grown forage crop species in the world, improved cultivars in alfalfa will certainly have a high economic impact.

Limitations and future directions

Despite the popularity and importance of the crop, genetic and genomic resources for tetraploid alfalfa are meager which limits advanced analyses. For instance, there are few sequence data processing pipelines that support *de novo* SNP calling. Since the species does not have a genome reference, we have to align the tag sequences to the *M. truncatula* (diploid relative) genome for chromosome assignment, but only about 60% of the SNPs were aligned to *Medicago* genome and we were unable to pinpoint the physical locations of the rest of the SNPs markers. The presence of SNPs with no information about the physical location poses challenges when distinguishing between sequencing errors from the true variants in alfalfa.

Being an autotetraploid exhibiting tetrasomic inheritance, genetic linkage mapping in alfalfa is cumbersome and limited to markers segregating in certain patterns. There are currently no mapping programs or software packages designed specifically for autotetraploid that can handle thousands of markers. In this study, the single dose alleles (SDA) SNPs (Aaaa x aaaa) specific to one parent were used for genetic linkage mapping which represent only a portion of

the entire genome. Moreover, mapping QTLs in an F1 population, captures variation within each parent rather than variation between parents. Therefore, the QTLs we identified in this study may not represent the entire set of causal loci for the traits we mapped. Furthermore, the QTLs reported in these studies for different alfalfa traits explained relatively lower portions of the total on phenotypic variations ($R^2 < 0.20$) and increasing the precision with increasing the number of locations may be warranted.

The phenotyping process, especially the visual rating methods we used in this research could generate some bias in the data. Although, the same individuals recorded visual scores, yet the data collected between environments and between seasons could bear some human bias. The visual rating method was also time consuming. Therefore, switching to newer technology for high-throughput field phenotyping of alfalfa traits such as winter killing and fall regrowth height could be more effective. Generating uniform alfalfa clones from stem cutting for the indoor freeze test is labor intensive because only a part of clones showed uniformity due to environmental or possible epigenetic variations. Therefore, we grew several clones in the greenhouse to select only the uniform ones for freezing test. In addition, the clones varied in growth depending on the vigor of the source plant, indicating that environmental variation must be addressed while cloning alfalfa.

Despite the limitations associated with alfalfa genome complexity, genomic resources and tools, phenotyping methods and alfalfa clonal uniformity, this study provides a foundation for further investigations and buildup of genomic resources for future alfalfa improvement programs. The genetic linkage maps are one of the most saturated alfalfa genetic maps published so far and may offer a framework for the future linkage mapping and allow comparative marker analysis with respect to grouping and positions within the linkage groups. Moreover, other traits

segregating in the population can also be mapped on the same genetic linkage map. For instance, we have a plan to map QTL for freezing tolerance after completion of the freezing tests. This will allow comparing the genetic relationship between the QTLs detected for winter hardiness phenotypes in the field and indoor freezing tolerance phenotype. The sequences of SNP markers identified for traits of interest can be used for putative homolog detection using the BLAST search either on *M. truncatula* genome or on the genome of CADL (cultivated alfalfa at diploid level). In this study, we have provided putative homologous genes related to flowering time, yield and leaf rust, but not for fall dormancy and winter hardiness. Relationships between the markers identified in the study and putative candidate genes for the traits of interest can be searched. For example, a search for *CBF* (C-repeat binding factor) gene which has been identified as the most important genes encoding proteins for cold acclimation can be initiated in alfalfa with the SNPs we identified for winter hardiness QTLs.

Improvement of alfalfa via heterotic hybrid breeding method is almost impossible because of self-incompatibility and inbreeding depression. However, exploiting partial heterosis by developing limited generations of synthetic cultivars is a common breeding method that can be applied in such forage species. By far, the most explored alfalfa improvement method is recurrent selection (RS), which is used to develop alfalfa synthetic cultivars by increasing the frequency of desired alleles for the target traits. Marker-assisted selection (MAS) is important to improve alfalfa traits like fall growth, winter hardiness and biomass by shortening breeding cycles and reducing their numbers compared to the RS. Marker-assisted backcrossing (MAB) is often performed to incorporate one or few major genes or QTLs into elite lines while marker-assisted pyramiding (MAP) can be done to combine several genes in an individual genotype. In the next step of this project we can incorporate the QTLs with relatively higher effects on

phenotypes such as dormancy QTL (ndorm1) and winter hardiness QTL (wh1) using MAP to enhance fall regrowth and winter hardiness of elite germplasm.

However, the validation of these QTLs will be essential before adopting them in MAS to ensure the loci are stable and repeatable across populations and environments. The non-dormant and winter-hardy F1 plants can be selected and polycrossed to develop F2 validation populations. If the QTLs identified for higher fall regrowth and winter hardiness are present in the segregating F2 progeny, these QTL markers can be used in MAB or MAP. The identified QTLs can also be verified with genome-wide association studies (GWAS) using diverse germplasm. We have access to a GWAS panel in the forage breeding program that was established in 2013 and has been evaluated for several traits including fall dormancy and winter hardiness. The panel will be genotyped and used for further linkage analysis and validation of the QTLs identified in bi-parental mapping. If GWAS shows the trait associated SNPs in the corresponding regions where QTLs were identified, then we can narrow down the candidate genomic regions and confirm the markers for breeding programs. The stable QTL markers for the traits of interest will be valuable genomic resources for future alfalfa improvement initiatives.